

# Diagnostic application of total antioxidant capacity in seminal plasma to assess oxidative stress in male factor infertility

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## Abstract

**Purpose** This study was undertaken in order to establish a new reference value for the total antioxidant capacity (TAC) in seminal plasma as a predictor of fertility. This study also aims to propose a detailed protocol for the TAC assay including calculation of assay results and assessment of sensitivity and specificity over possible cutoff values in infertile men and controls with proven and unproven fertility.

**Methods** Seminal plasma from 279 infertile patients and 46 normal healthy men referred to a male infertility testing laboratory were tested to measure TAC by a colorimetric assay kit. Receiver operating characteristics (ROC) curves were generated to establish cutoff values, sensitivity, and specificity, and the distribution of cutoff values in controls and infertile patients was calculated.

**Results** Infertile patients showed significantly lower levels (mean ± SEM) of total antioxidants (micromolar Trolox equivalents) in their seminal plasma ( $1863.84 \pm 27.16 \mu\text{M}$ ) compared to those from fertile men ( $2013 \pm 56.04 \mu\text{M}$ ,  $P=0.019$ ). A preferred cutoff TAC value of  $1947 \mu\text{M}$  could facilitate better

diagnosis of oxidative stress (OS) in men with male factor infertility. At this threshold, the specificity of TAC assay was 63.0 % and the sensitivity 59.5 % with a positive predictive value of 90.7 % and a negative predictive value of 20.4 %.

**Conclusions** Our results establish a new diagnostic cutoff TAC value of  $1947 \mu\text{M}$  in seminal plasma to distinguish prevalence of OS in infertile patients compared to healthy men. This study provides a robust reference value of seminal plasma TAC that may provide an important diagnostic tool to the physicians for managing OS and male factor infertility in such patients.

**Keywords** Male infertility · Oxidative stress · Seminal plasma · Total antioxidant capacity · Diagnostic value · Specificity and sensitivity

## Introduction

Male infertility factors are present in about 20 % of infertile couples and contributory in about 30 to 40 %. An infertility evaluation should be performed if a couple has not achieved conception after 1 year of unprotected intercourse [1]. Oxidative stress (OS) is a common pathology in approximately half of all infertile men and is associated with pathogenesis of sperm DNA damage and dysfunction [2–6]. It results in negative changes in semen parameters such as sperm concentration, motility and morphology, and eventually male factor infertility [7].

Spermatozoa rely on oxidative phosphorylation in the mitochondria and glycolysis in the head and principal piece of the flagellum for energy. Oxidative phosphorylation is coupled with respiratory (electron transport) chain—a process accompanied by reactive oxygen species (ROS) generation [8]. ROS are natural products of cellular metabolism which, in physiological amounts, are essential for sperm processes such as capacitation, hyperactivation, and acrosome reaction

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**Capsule** Our results establish a new diagnostic cutoff TAC value of  $1947 \mu\text{M}$  in seminal plasma to distinguish prevalence of OS in infertile patients compared to healthy men.

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leading to successful fertilization [9, 10]. Immature spermatozoa with excessive cytoplasm around the mid piece is also a concerning source of ROS resulting in impaired motility and abnormal morphology that impacts negatively on its fertilization potential [11]. In addition, seminal leukocytes are capable of producing up to 1000 times more ROS than immature spermatozoa causing high OS [12, 13]. Spermatozoa have limited antioxidant defense mechanisms and low concentration of ROS scavenging enzymes [8]. Also, spermatozoa are rich in polyunsaturated fatty acids and therefore susceptible to ROS attack [14]. However, seminal plasma has a very effective antioxidant defense system that provides spermatozoa with a protective environment against OS [15]. Antioxidants can protect cells against OS via three mechanisms: prevention, interception, and repair [16, 17]. OS occurs when there is excessive ROS production and limited antioxidant scavenging capacity resulting in an imbalance [18–21]. OS has been implicated in several male infertility-associated pathologies, including leukocytospermia, varicocele, as well as idiopathic infertility [22–24]. Our group has recently proposed a reference value and range of seminal ROS [25, 26]. Still there is a lack of complete clarity concerning the guidelines for determination of what is excessive ROS production or limited antioxidant capacity. It is therefore clinically important to establish the reference values for measuring the total antioxidant capacity (TAC) in the seminal plasma.

In this study, our goal was to establish a reliable and robust cutoff value of TAC in seminal plasma by investigating a large sample size. We established the cutoff value, including assay sensitivity, specificity, and negative and positive predictive values, for better diagnosis and management of OS in male factor infertility patients.

## Materials and methods

### Subjects

The study was approved by the Institutional Review Board of the Cleveland Clinic, and all the patient as well as donor samples used in the study were obtained with informed consent. Semen samples were used from 279 infertile patients referred to our andrology lab and 46 healthy controls with proven and unproven fertility screened from June 2014 through October 2015. The infertile men attending our male infertility clinic had confirmed male factor infertility after at least 1 year of unprotected intercourse. All patients were evaluated by a male infertility specialist. Patients with azoospermia, severe oligozoospermia, retrograde ejaculation, and collection problems (partial ejaculate) were excluded. The female partners of these patients underwent a complete gynecological investigation and were found to be healthy.

The controls consisted of healthy male volunteers selected on the basis of normal semen analysis ( $n=21$ ) according to the 2010 World Health Organization guidelines [27] or were of proven fertility ( $n=25$ ). All the male volunteers were tested for leukocytospermia and those men with  $>1.0 \times 10^6/\text{mL}$  white blood cells in semen as detected by myeloperoxidase or Endtz test were excluded from the study. Only 20.07 % (56/279) of infertile patients were identified with leukocytospermia and hence not grouped separately.

### Semen collection and preparation

- (i) Semen specimens were collected by masturbation after 48 to 72 h of sexual abstinence. After complete liquefaction at 37 °C for 20 min, a 5- $\mu\text{L}$  aliquot of each specimen was loaded on a 20- $\mu\text{M}$  MicroCell chamber (Vitrolife, San Diego, CA) where it was analyzed for sperm concentration and motility.
- (ii) All samples were centrifuged at  $300 \times g$  for 10 min at room temperature. Clear seminal plasma was aliquoted and frozen at  $-80$  °C until the time of TAC assay.

### TAC assay

Seminal plasma TAC measurement was done using the antioxidant assay kit and reagents (Cat #709001; Cayman Chemical, Ann Arbor, MI). The principle of the assay is based upon the ability of all antioxidants in the seminal plasma to inhibit the oxidation of the 2,2'-azino-di-[3-ethylbenzthiazoline sulfonate] (ABTS) to  $\text{ABTS}^+$  resulting in change 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 (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water-soluble tocopherol analog. Results are reported as micromoles of Trolox equivalent.

The steps followed in the assay are given below.

- (i) All reagents and samples were equilibrated to room temperature at least 30 min prior to the beginning of the assay. Trolox standards and reagents were prepared as per the manufacturer's instructions at the time of the assay.
- (ii) The antioxidant assay buffer concentrate was diluted 1:9 in a 15-mL conical tube. The reconstituted vial is stable for 6 months when stored at 4 °C.
- (iii) The lyophilized metmyoglobin powder was reconstituted with 600  $\mu\text{L}$  of assay buffer. It is adequate for 60 wells. The reconstituted reagent is stable for 1 month when stored at  $-20$  °C.
- (iv) One milliliter of reconstituted lyophilized Trolox was used to prepare the standard curve. It is stable for 24 h at 4 °C.

- (v) The working solution of 441  $\mu\text{M}$  was prepared by two serial dilution, first by adding 10  $\mu\text{L}$  of 8.82 M hydrogen peroxide to 990  $\mu\text{L}$  of ultrapure water and then further by removing 20  $\mu\text{L}$  and diluting with 3.98 mL of ultrapure water. It is stable for 4 h at room temperature.
- (vi) Chromogen (containing ABTS) was reconstituted with 6 mL of ultrapure water which was sufficient for 40 wells. The reconstituted vial is stable for 24 h at 4  $^{\circ}\text{C}$ . It is light sensitive and was prepared in indirect light.
- (vii) All the frozen samples of seminal plasma were thawed and centrifuged at  $300\times g$  for 7 min to ensure precipitation of any cellular component such as spermatozoa or cell debris, and the supernatant was used. Each sample was diluted 1:9 with assay buffer in clear microfuge tubes to avoid variability because of interference by the plasma proteins or sample dilution. All the samples were analyzed in duplicate.
- (viii) Samples as well as Trolox standards that were added to each well in duplicate were noted down using a plate template.
- (ix) Ten microliters of Trolox standard and test samples were loaded into the corresponding wells of a 96-well plate and assayed in duplicate (Cayman Chemical, Ann Arbor, MI).
- (x) Ten microliters of metmyoglobin and 150  $\mu\text{L}$  of chromogen were added to all standard/sample wells. The reaction was initiated by adding 40  $\mu\text{L}$  of hydrogen peroxide as quickly as possible.
- (xi) The plate was covered and incubated for 5 min on a horizontal plate shaker at room temperature (Eppendorf MixMate, Hamburg, Germany).
- (xii) Absorbance was monitored at 750 nm using a microplate reader (BioTek Instruments, Inc., Winooski, VT).

### Calculation of assay results

- (i) 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 ( $\mu\text{M}$  of Trolox equivalent) was plotted for the standard curve in each run, from which the unknown samples were determined (Fig. 1).
- (ii) 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:

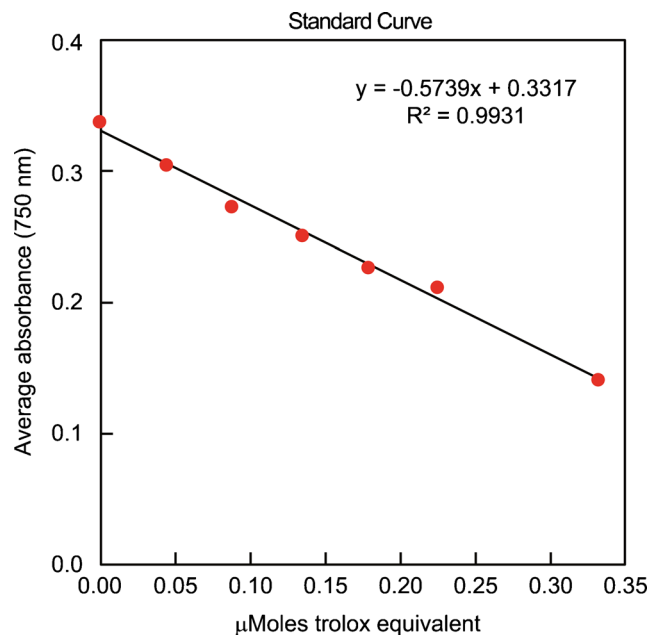
$$\text{Antioxidant}(\mu\text{M}) = [(\text{Unknown average absorbance} - Y\text{-intercept}) \div \text{Slope}] \times \text{dilution} \times 1000$$

### Statistical analysis

The difference in distributions of TAC levels between infertile patients and healthy donors was assessed using the Wilcoxon rank sum test or chi-square test. Summaries of the distributions include frequency (%) and mean  $\pm$  standard error of the mean (SEM). A receiver operating characteristics (ROC) curve was plotted to display estimated sensitivity, specificity, positive predictive value, negative predictive value, and accuracy 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.

### Results

The background information on semen parameters in healthy donors ( $n=46$ ) and infertile patients ( $n=279$ ) used in the study is presented in Table 1. In the group of healthy men, the age was  $37.57 \pm 11.37$  years, the ejaculatory abstinence was  $3.54 \pm 1.72$  days, and the sperm concentration was  $54.50 \pm 33.53 \times 10^6/\text{mL}$  expressed as mean  $\pm$  standard deviation. In



**Fig. 1** Example of a standard curve for TAC measurement: standard Trolox concentrations are represented on the X-axis and the absorbance on the Y-axis

**Table 1** Background information on semen parameters in healthy donors and infertile men used in the study

Parameter	Healthy donors ( <i>n</i> =46)	Infertile patients ( <i>n</i> =279)
Age (years)	37.57 ± 11.37	35.61 ± 6.06
Ejaculatory abstinence (days)	3.54 ± 1.72	3.98 ± 2.75
Sperm concentration (×10 <sup>6</sup> /mL)	54.50 ± 33.53	47.27 ± 53.58
Leukocytes (×10 <sup>6</sup> /mL)	0	0.27 ± 0.89
Diagnosis of varicocele (%)	0	34.05

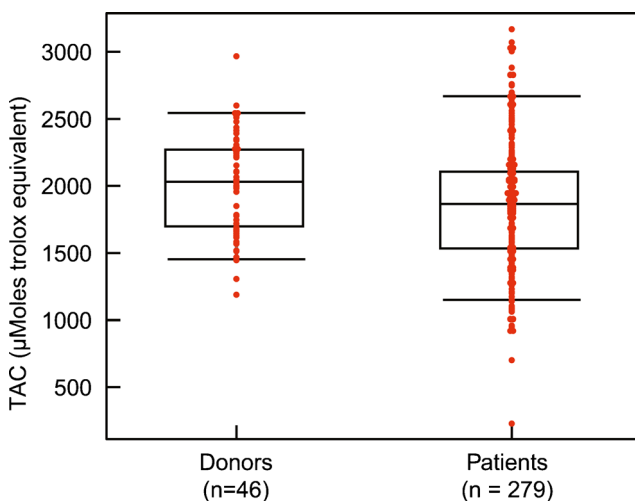
Data are expressed as mean ± standard deviation

the group of infertile patients, the age was 35.61 ± 6.06 years, the ejaculatory abstinence was 3.98 ± 2.75 days, and the sperm concentration was 47.27 ± 53.58 × 10<sup>6</sup>/mL. Among the infertile patients, the level of leukocytes was 0.27 ± 0.89 × 10<sup>6</sup>/mL and 34.05 % (95/279) men presented with a varicocele.

Distribution of TAC levels between healthy donors and infertile patients is shown in Fig. 2. A total of 325 TAC measurements were conducted in order to associate TAC levels with status of a subject, as either a healthy donor (control) or an infertile patient. The mean ± SEM TAC values were 2013 ± 56.04 μM in healthy donors as compared to 1863.84 ± 27.16 μM in infertile patients (*P*=0.019).

In our study, an ROC curve was plotted to determine cutoff value, sensitivity, and specificity of the TAC assay. A preferred cutoff value of 1947 μM could facilitate better diagnosis of OS in men with male factor infertility. At this cutoff point, the sensitivity was 59.5 %, specificity 63.0 %, positive predictive value 90.7 %, and negative predictive value 20.4 %. The accuracy of the test was 60.0 % (AUC=0.608) (Fig. 3).

The status of a subject was also determined either as a healthy donor or an infertile patient depending upon two potential cutoff settings for antioxidants: (a) TAC levels higher than 1790 μM and (b) TAC levels higher than 1947 μM. For

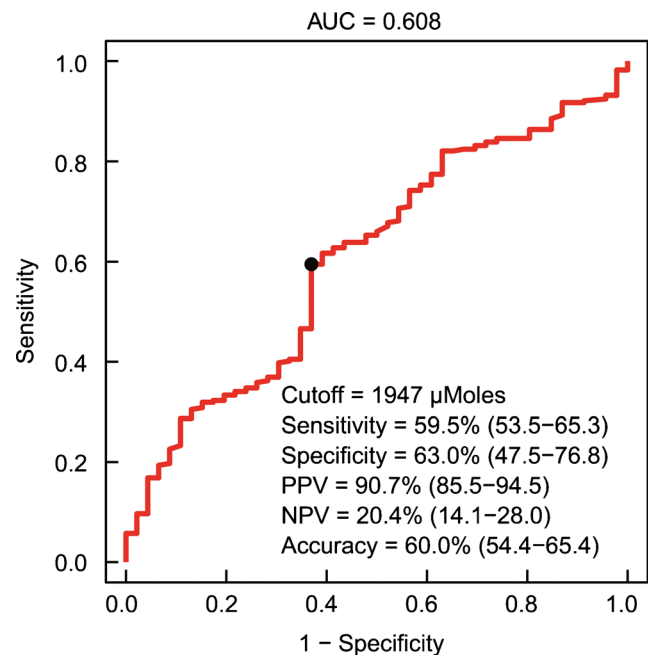


**Fig. 2** Distribution of TAC levels between healthy donors (*n*=46) and infertile patients (*n*=279)

the potential cutoff level higher than 1790 μM, healthy donors as well as infertile patients did not show any significant difference (*P*=0.46); 65.2 % donors (*n*=30) and 59.5 % patients (*n*=166) presented with more than this TAC level. On the other hand, a significantly higher proportion (*P*<0.05) of healthy donors (63 %; *n*=29) presented the second potential cutoff value for TAC levels higher than 1947 μM in comparison to that of infertile patients (40.50 %; *n*=113). These findings led to the establishment of seminal plasma TAC levels higher than 1947 μM as the preferred cutoff value.

## Discussion

The variability in TAC levels among infertile patients as shown in Fig. 2 can be explained in terms of varying degrees of OS [28] or any other clinical diagnosis such as varicocele. The background information on semen parameters in the



**Fig. 3** Receiver operating characteristics (ROC) curve showing the area under curve (AUC), cutoff, sensitivity, specificity, positive predictive value, negative predictive value, and accuracy of the assay

infertile patient population as presented in Table 1 revealed that 34.05 % (95/279) men presented with a varicocele. Wang et al. reported that a significant portion of TAC has a genetic component [29]. How this relates to OS and/or TAC and their role in infertility is not yet understood. However, such information will be very useful to the clinicians in their decision-making for treating these infertility cases.

The impact of factors such as paternal age as well as the rate and time of centrifugation on seminal oxidative stress has been investigated. In the present study, the average age of healthy men was  $37.57 \pm 11.37$  years, while it was  $35.61 \pm 6.06$  years in the case of infertile patients. Cocuzza et al. demonstrated that ROS levels are significantly higher in seminal ejaculates for fertile older men ( $\geq 40$  years) compared to younger ones ( $< 40$  years). In the category of fertile older men, the average age was 43.5 years, whereas the average age of fertile younger men was 33.5 years. The average age of infertile men who served as positive control was 42.65 years in the case of older men, whereas it was 27.95 years in the case of younger men [30]. On the other hand, a previous study by our group did not report any significant difference in seminal ejaculate ROS levels between older and younger infertile men. In this study, the average age of older infertile men ( $> 40$  years) was 46.6 years, followed by 35.3 years (31–40 years category) and 28.2 years ( $\leq 30$  years) in the groups of younger infertile men [31]. The effect of centrifugation rate and time ( $200 \times g$  for 2 or 10 min and  $500 \times g$  for 2 or 10 min) on the formation of ROS in semen of healthy and infertile men was investigated by Shekarriz and coworkers [32]. They concluded that the time of centrifugation was more important than  $g$ -force for inducing ROS formation in semen as the increase in ROS was less when semen was centrifuged for 2 as compared to 10 min. Bani Hani suggested that the rate of centrifugation can also influence seminal TAC levels, and noted a decrease in TAC upon raising the centrifugation force from 220 to  $400 \times g$  [33].

The impact of ROS has widespread implications in the genitourinary system, including sperm physiology, fertility evaluation, reproductive outcomes, and antioxidant therapy [34]. Although ROS is formed during normal enzymatic reactions, cellular damage is prevented by the antioxidant scavenging system via enzymatic and nonenzymatic antioxidant pathways [35, 36]. This finely balanced oxidant-antioxidant system allows the formation of beneficial oxidants for normal cellular functions and concurrently prevents the damaging effects of excess OS [34]. TAC assesses the cumulative effect of all antioxidants present within the semen, based on their ability to scavenge free radicals with any specific or nonspecific mechanism(s) available [37–39].

Although multiple tests are available to measure TAC [39], the Trolox equivalent antioxidant capacity assay and the ferric reducing ability of the seminal plasma are the two most widely used in recent times. The first method, also called as ABTS

assay, is based on the inhibition of oxidation of the ABTS to  $ABTS^+$ . The second, ferric reducing antioxidant power (FRAP) assay, is based on the formation of  $[Fe^{2+}]$  tripyridyltriazine from its oxidized  $[Fe^{3+}]$  form. These assays have used colorimetric techniques to measure the seminal TAC. Our laboratory standardized a colorimetric TAC assay in a kit form that was used successfully with seminal plasma [40].

The evaluation of seminal plasma TAC levels has been emphasized to decide on the prognosis and the diagnosis of male infertility, especially in idiopathic male infertility [41, 42]. These authors reported average TAC levels of  $3239 \pm 562.25$   $\mu M$  (expressed as mean  $\pm$  standard deviation) in healthy men (control) compared to  $1916.4 \pm 575.39$   $\mu M$  in asthenoteratospermic and  $1896.7 \pm 650.86$   $\mu M$  in oligoasthenoteratozoospermic infertility patients, respectively. However, in our study, infertile patients showed significantly lower ( $1863.84 \pm 27.16$   $\mu M$  expressed as mean  $\pm$  SEM) seminal plasma TAC levels compared with healthy men ( $2013 \pm 56.04$   $\mu M$ ;  $P = 0.019$ ). For seminal plasma TAC levels higher than 1947  $\mu M$ , the proportion of healthy donors was significantly more in comparison to infertile patients ( $P < 0.05$ ). The seminal plasma ROC curve suggested 60 % accuracy of the test with good sensitivity and specificity over a cutoff TAC value of 1947  $\mu M$ . It also indicated a high positive predictive value (90.7 %) and a low negative predictive value (20.4 %). The assay is reported to have an inter-assay coefficient of variation of 3 % and intra-assay coefficient of variation of 3.4 %. Such minimal variability suggests our TAC assay to be more robust and reliable and the cutoff value to be more precise that may help the clinicians in their decision-making to offer appropriate therapy to these patients.

ABTS assay has been used to measure the seminal plasma TAC for a variety of diagnostic purposes. In an earlier study, significantly higher TAC was reported in varicocele patients in comparison to patients with genitourinary inflammation or normal fertile controls, suggesting possible involvement of systemic hormones prolactin and tetraiodothyronine in the regulation of seminal TAC [43]. Further analysis between these hormones and seminal parameters showed an inverse correlation between prolactin and sperm motility, while a direct correlation of TAC with prolactin and tetraiodothyronine was observed [43]. The effect of spermatic vein ligation on seminal TAC in patients with varicocele has also been assessed by the same method. The evaluation of seminal TAC levels in these patients before and after surgery revealed that spermatic vein ligation can improve the seminal TAC in patients with middle- and high-grade varicocele [44]. They noted seminal plasma levels of  $2580 \pm 250$   $\mu M$  in preoperative grade I varicocele patients as against  $2610 \pm 160$   $\mu M$  after surgery. In varicocele grade II and III patients, the TAC levels were  $2360 \pm 230$   $\mu M$  in preoperative and  $2660 \pm 150$   $\mu M$  in postoperative cases. Further studies are needed to establish associations between such surgical procedures or hormones

with TAC scores and implications of such association in the management of infertility patients.

It is interesting to find that by using the same assay, higher seminal TAC levels were obtained after a shorter period of ejaculatory abstinence (1 vs. 4 days) before oocyte insemination for IVF, suggesting that it may protect sperm from OS-induced damage by a mechanism independent of lipid peroxidation of sperm membranes [45]. The same authors hypothesized that higher seminal TAC would reduce exposure of sperm to harmful ROS and may be a protective mechanism for improving sperm quality. They argued that purging sperm from the cauda of the epididymis or vas deferens by shortening the period of ejaculatory abstinence could diminish the population of senescent sperm, thus reducing ROS and perhaps lessening the protective effect of TAC [45]. Another study reported a positive correlation of the magnitude of decrease in the seminal plasma TAC levels with the abnormal semen parameters such as concentration, motility, and morphology in men with idiopathic infertility [46]. They suggested TAC levels of  $1098 \pm 120 \mu\text{M}$  in oligozoospermic patients compared to  $1267 \pm 75 \mu\text{M}$  in normozoospermic men.

Many environmental factors are known to affect fertility. How these are linked to OS and TAC in the semen samples is not clear. Kumar and colleagues measured TAC values in the seminal plasma of health workers occupationally exposed to ionizing radiation using the ABTS assay and reported a positive correlation of TAC with sperm chromatin integrity [47]. Recently, this technique was used to measure seminal TAC in males environmentally exposed to lead ( $1470 \pm 240 \mu\text{M}$  in low-exposure group vs.  $1320 \pm 170 \mu\text{M}$  in high-exposure group). It was reported that lead exposure induces OS in seminal plasma and modulates antioxidant defense systems [48]. Similarly, many other environmental issues linked to male factor infertility and their prevention/treatment could benefit from such TAC evaluation of semen samples collected in a clinical lab or at the exposure sites.

Any correlation of TAC with antioxidant therapy may also play a useful role in such patient care. The supplementation of antioxidant alpha-lipoic acid (also called thioctic acid) in infertile men has been shown to result in a significant increase in seminal TAC levels in comparison to placebo, suggesting this as an oral antioxidant therapy of asthenoteratospermia [49]. Raigani and colleagues studied the impact of zinc sulfate and folic acid supplementation on seminal plasma TAC levels and sperm functional parameters in oligoasthenoteratozoospermic men [50]. However, no statistically significant differences in seminal TAC could be detected before 16 weeks of randomized double-blind treatment.

A recent prospective, randomized, controlled clinical tamoxifen treatment in idiopathic oligoasthenospermic patients resulted in increased serum and seminal plasma TAC and, significantly, spermatozoa intracellular ROS, indicating the antioxidant role of tamoxifen [51]. From the abovementioned

studies, it is evident that the seminal plasma TAC assay has assumed importance as a component of clinical trials in the prevention of OS-related male factor infertility as seminal TAC can provide useful information [49–51]. As such, results of such trials are expected to have wide clinical importance in fertility clinics and laboratories, thereby establishing the practical utility of the TAC assay.

The FRAP assay has also been used widely for measurement of seminal plasma TAC levels. FRAP is a ferric-reducing agent with antioxidant potential that measures change in absorbance at 593 nm due to the formation of a blue-colored  $[\text{Fe}^{2+}]$  tripyridyltriazine compound from colorless oxidized  $[\text{Fe}^{3+}]$  form, by the action of electron-donating antioxidants [42]. Using this agent in a simple colorimetric method, they reported significantly lower seminal plasma TAC levels in oligoasthenozoospermic ( $1389.72 \pm 242.11 \mu\text{M}$ ), asthenozoospermic ( $1777.88 \pm 239.87 \mu\text{M}$ ), and azoospermic ( $1702.67 \pm 485.95 \mu\text{M}$ ) patients than in seminal plasma of normozoospermic ( $1980.82 \pm 160.58 \mu\text{M}$ ) men.

Recently, Layali and colleagues have reported lower TAC levels ( $1230.25 \pm 352 \mu\text{M}$ ) in hyperviscous semen samples as compared to nonhyperviscous semen samples ( $1710.31 \pm 242.11 \mu\text{M}$ ) [52]. They attributed this to a severe impairment of seminal antioxidant systems and an increased sperm membrane lipid peroxidation in patients with hyperviscous semen. Increased ROS in the seminal plasma of patients with hyperviscous semen, originating from leukocytes and abnormal sperm, may decrease the effective TAC and increase the harmful effects of ROS [52]. A severe impairment of the high and low molecular weight antioxidants in semen of patients with hyperviscous semen has also been reported [53]. However, in the present study, we did not include hyperviscous semen samples as a separate group. Significantly higher TAC levels have been reported in azoospermia ( $2913 \pm 900 \mu\text{M}$ ) than in teratozoospermia ( $2269 \pm 570 \mu\text{M}$ ) or the normal controls ( $2120 \pm 900 \mu\text{M}$ ) [54]. This relationship needs to be investigated further.

Another study by the same group suggested significantly higher TAC levels in normozoospermic ( $1672 \pm 631 \mu\text{M}$ ) and oligozoospermic ( $1763 \pm 561 \mu\text{M}$ ) men compared to fertile men ( $1065 \pm 377 \mu\text{M}$ ) [55]. The seminal TAC levels even in normal fertile men in these two studies by the same group were different. It could be due to the lack of standardization in the TAC assay unlike in our present study. Those authors supported their findings by suggesting that TAC reflects the effect of low molecular weight antioxidants but not the activity of endogenous antioxidant enzymes (e.g., catalase, SOD, etc.) or metal-binding proteins. Kratz et al. further suggested that such inconsistency in the TAC values of infertile patients might be caused by different study conditions and that any increase in the antioxidant capacity of male semen may not necessarily lead to improved sperm parameters or the subsequent fertilization process [55]. Recently, Yousefniapasha and

colleagues also used this assay to measure the seminal plasma TAC of infertile men with history of smoking and showed higher TAC levels in their semen ( $1785.45 \pm 718.18 \mu\text{M}$ ) [56]. They suggested that the increased nitric oxide levels associated with smoking might exceed the capacity of the antioxidant defense system leading to increased oxidative damage and decreased fertility and thus associated with higher TAC levels. This needs further investigation.

Our group previously measured ROS production and TAC levels by chemiluminescence assay to create a ROS-TAC score to provide to the clinicians with a means to suggest early varicocele repair for patients and prevent further sperm damage by OS [57]. Infertile patients with varicocele had lower semen quality scores, higher ROS, and lower TAC and ROS-TAC scores compared to healthy men. TAC levels of  $1186 \pm 96.9 \mu\text{M}$  were noted in infertile varicocele patients, while the levels were  $1443 \pm 105 \mu\text{M}$  in healthy donors (control) and  $939 \pm 107 \mu\text{M}$  in patients with varicocele [57]. Semen quality scores were derived from concentration, motility, curvilinear velocity, straight-line velocity, average path velocity, amplitude of lateral head displacement, linearity, and morphology of spermatozoa. Semen and ROS-TAC scores serve as good indicators about fertilizing potential because an imbalance between ROS production and TAC in seminal plasma leads to OS-induced male infertility [58]. Pasqualotto and coworkers also suggested that the ROS-TAC score may be more predictive of subfertility than TAC or ROS levels alone as it gives the benefit of measuring two reliable parameters [57]. In the present study, we established a new cutoff value of seminal plasma TAC by investigating a large number of healthy donors as well as infertile patients. By plotting the ROC curve over a range of possible cutoff points, the sensitivity, specificity, and negative and positive predictive values have been estimated with minimal variability to distinguish between healthy men and male factor infertility patients. A cutoff value of  $1947 \mu\text{M}$  seminal plasma TAC was chosen to maximize the sum of sensitivity and specificity.

One limitation of our study was that the subjects were grouped either as healthy donors (based on normal semen analysis of proven fertile as well as normozoospermic men according to WHO 2010 guidelines) or as infertile patients (attending a tertiary care hospital). Only some of the donors were of proven fertility, and hence, we did not include proven fertile men as a separate group in our study. Secondly, although the leukocytospermic men were excluded in the group of healthy donors, only a limited number of infertile patients were leukocytospermic and hence not included as a separate group. Furthermore, healthy donors (control group) had a smaller sample size in comparison to infertile patients. Another limitation was that except for varicocele, we did not examine any other clinical diagnosis of our infertile population regarding the exact etiology of infertility or reproductive potential in these patients.

## Conclusions

In conclusion, we have provided a detailed protocol to measure seminal antioxidant levels and established the reference values below which the TAC levels are abnormal and may result in OS in the presence of high ROS. The results of the present study establish a new diagnostic cutoff value of  $1947 \mu\text{M}$  seminal plasma TAC to distinguish between healthy donors and male factor infertility patients. Each laboratory must establish their own reference values using the protocol described. This study provides a reference value taking into account a large number of infertile patients, which will help in better diagnosis and management of male factor infertility.

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