

Poor Semen Quality and ROS-TAC Scores in Patients with Idiopathic Infertility

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Key Words

Infertility · Semen · Spermatozoa · Oxidative stress · Antioxidants

Abstract

Introduction: To compare the semen quality score and the seminal oxidative stress reactive oxygen species (ROS) and total antioxidant capacity (ROS-TAC score) in men with idiopathic infertility with normal donors and to a known group of fertile and infertile men. **Material and Methods:** Principal component analysis was applied to provide a standardized score in 36 men with idiopathic infertility and 19 controls attending our infertility clinic. A logistic regression analysis comparing the fertile and infertile men was used. **Results:** Compared to controls, patients with idiopathic infertility had significantly lower sperm concentration, sperm motility and normal morphology ($p < 0.05$) and lower semen quality scores (83.0 ± 14.5 vs. 100.0 ± 10.0 ; $p < 0.001$). Compared to controls, the ROS levels were higher in the idiopathic infertility group (2.3 ± 0.21 vs. 1.3 ± 0.3 ; $p = 0.006$), whereas the TAC levels were lower in the idiopathic infertility ($1,014.75 \pm 79.22$ vs. $1,653 \pm 115.29$; $p = 0.001$). Idiopathic infertility patients had lower ROS-TAC scores (32.8 ± 14.2) than controls (50.0 ± 10.0) ($p < 0.001$). 64% of men with idiopathic infertility will remain infertile during 1-year follow-up. **Conclusions:** Patients with idiopathic infertility have lower scores of semen quality and ROS-TAC.

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Introduction

In some cases of couple's infertility, standard investigations reveal no detectable abnormalities, and therefore the couple is diagnosed with unexplained or idiopathic infertility [1, 2]. Even though the semen analysis represents one of the cornerstones in the investigation of the infertile male, the clinical value of traditional semen parameters in the diagnosis of male infertility is the subject of considerable debate [2–7]. Semen analysis typically produces a wide variety and number of semen characteristics that are correlated, indicating that underlying measures of semen quality can be used to reduce the number of variables evaluated [8–11]. Computer-assisted semen analyzer (CASA) refers to an automated system (hardware and software) to visualize and digitize successive images of sperm, process and analyze the information, and provide accurate, precise, and meaningful information on the kinematics of individual cells, and also population summary statistics, that is, mean values. The authors emphasized that there was useful information in measures of vigor and pattern of sperm motion (e.g., cur-

The present study was jointly undertaken by the Glickman Urological Institute, The Cleveland Clinic Foundation, Cleveland, Ohio, USA, and the Institute of Biotechnology, University of Caxias do Sul, RS, Brazil.

vilinear velocity, average path velocity, and linearity) as well as percentage of motile sperm. Some of the variables evaluated with the CASA machine are the curvilinear velocity (VCL), straight line velocity (VSL), average path velocity (VAP), linearity (LIN), and lateral head displacement (ALH) [12].

Therefore, after the introduction of CASA, the number of sperm characteristics examined has increased to the extent that each semen evaluation quantifies nine semen characteristics [10, 13, 14]. Although these characteristics are unique measures of semen quality, they are not independent of one to another in the sense that patients with low motility tend to have low concentration and vice versa. Since many of these characteristics are interrelated, an overall semen score can be developed by appropriate statistical models [15].

Despite the presence of numerous tests of sperm quality and function, no single laboratory test can determine with accuracy and precision whether a man is fertile [3, 9, 11]. In fact, impaired sperm function is an obvious and general cause of male infertility.

There are assays that may be performed in men with idiopathic infertility, such as the hypoosmotic swelling test (HOST) as well as the acrosin activity assay. In fact, the percentage of swollen spermatozoa and acrosin profiles are significantly lower in the infertile men than in the fertile donors. Considering the lowest values of the outcome of the HOST and the acrosin activity assay in the group of fertile men as the lowest normal values, it has been proved according to a previous study that HOST and acrosin activity assay could identify subpopulations of infertile men of 37 and 26%, respectively. Therefore, these results support the employment of the HOST and the acrosin activity assay in the evaluation of idiopathic infertile men [16].

When the hyperactivated motility characteristics are compared in samples with normal and abnormal semen analyses, the total percentage of spermatozoa with hyperactivated motility are significantly lower in the group with abnormal semen analysis. The data indicate that lower hyperactivated motility of spermatozoa was found in patients with a score of zero for SPA and in patients with abnormal semen analysis. Therefore, although no direct correlations were found between the results of SPA and hyperactivated motility, evaluating hyperactivated motility may still be useful as an early indicator of capacitation abnormalities of human spermatozoa not measured by SPA [17].

Reactive oxygen species (ROS) are oxygen-derived molecules that act as powerful oxidants. ROS, such as su-

peroxide anion (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH^-), are formed as intermediary products in low concentrations in the male and female genital tracts [5–7]. ROS have the ability to react with any molecule and modify it oxidatively, resulting in structural and functional alterations.

Free radicals are important in both the normal function and the pathophysiology of human spermatozoa. It is now recognized that low, controlled levels of extracellular ROS produced by spermatozoa are involved in sperm capacitation and acrosome reaction. The mechanism by which ROS regulates these processes is unclear, but may involve tyrosine phosphorylation of sperm proteins. In fact, human spermatozoa rely on reduction-oxidation processes for normal functions such as hyperactivation, capacitation, acrosome reaction, and acquisition of sperm-fertilizing ability [5–7]. Even though the controlled generation of ROS in spermatozoa is associated with normal physiological functions, uncontrolled and excessive production of ROS however appears to have a significant role as one of the major factors leading to an infertile status [2, 3, 5–7, 18–23].

Excessive ROS production causes oxidative stress, resulting in decreased sperm motility, viability, and increased mid-piece sperm defects which impair sperm capacitation and acrosome reaction [19, 20].

Capacitation is a term used to define a complex and not well-characterized process that allows spermatozoa to complete their preparation to fertilize oocytes. Spermatozoa from many species incubated under specific conditions have the ability to produce small amounts of ROS without harming cell function and rather promoting signal transduction pathways associated with capacitation. The role of ROS as regulators of protein tyrosine phosphorylation has been known for a decade, but novel phosphorylations, such as those of PKA substrates, of MEK-like proteins, and of proteins with the threonine-glutamine-tyrosine motif, were recently evidenced [21, 22].

Human spermatozoa are rich in polyunsaturated fatty acids and are therefore susceptible to ROS attack [20]. To counteract the harmful effects of ROS, sperm and seminal plasma possess a number of antioxidant systems that scavenge ROS and prevent internal cellular damage [23–31]. The imbalance between ROS production and total antioxidant capacity (TAC) in seminal fluid indicates oxidative stress and is correlated with male infertility [25]. A composite ROS-TAC score may be more strongly correlated with infertility than ROS or TAC alone [7].

The purpose of our study was to compare the semen quality score and the seminal oxidative stress (ROS-TAC score) in men with idiopathic infertility with normal donors (controls) and to a known group of fertile and infertile men treated for their infertility.

Material and Methods

Principal component analysis was applied to nine semen characteristics (concentration, motility, morphology, and sperm motion characteristics assessed by CASA) to provide a standardized score in 36 men with idiopathic infertility and 19 controls attending our infertility clinic. A logistic regression analysis comparing the fertile ($n = 13$) and infertile ($n = 39$) men (treated male factor cases) was used to provide estimates of fertility based on the ROS-TAC score.

Semen Analysis

Computer-assisted semen analysis was performed on all specimens, with a Motion Analysis VP 50 semen analyzer (Motion Analysis Corp., Santa Rosa, Calif., USA). For each measurement, a 5- μ l aliquot was loaded on a counting chamber (MicroCell; Conception Technologies, La Jolla, Calif., USA). Four to eight representative fields containing 200 or more spermatozoa were examined. Samples were analyzed for concentration, percent motility, and complex motion characteristics. To ensure the accuracy of the CASA results, a manual assessment was also performed.

White Blood Cells

The presence of granulocytes in semen specimens was assessed by a myeloperoxidase test. A 20- μ l volume of liquefied specimen was placed in a Corning 2.0-ml cryogenic vial (Corning Costar Corp., Cambridge, Mass., USA): 20 μ l of phosphate-buffered saline (PBS, pH 7.0) and 40 μ l of benzidine solution were added. The mixture was vortexed and allowed to sit for 5 min. Five microliters of the specimen was placed on a Makler chamber (Sefi Medical, Haifa, Israel) and examined for cells that stained dark-brown and were therefore positive for neutrophils. Leukocytospermia was defined as the presence of at least 1×10^6 WBC/ml. In our study, we included both patients with ($n = 2$) and without leukocytospermia ($n = 34$).

Reactive Oxygen Species

Aliquots of liquefied semen were centrifuged at 300 g for 7 min. Seminal plasma was aliquoted and frozen at -20°C for later measurement of total antioxidant levels. The sperm pellet was washed twice with PBS, pH 7.4, and resuspended in the same medium at a concentration of 20×10^6 sperm/ml. ROS production was measured by the chemiluminescence assay method, using luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma Chemical Co., St. Louis, Mo., USA) as the probe. 10 μ l of 5 mM luminol prepared in dimethyl sulfoxide (Sigma Chemical Co.) was added to 400 μ l of the washed sperm suspension. ROS levels were determined by measuring chemiluminescence with a luminometer (LKB 953, Wallac Inc., Gaithersburg, Md., USA) in the integrated mode for 15 min, and results were expressed as 10^4 counted photons per minute (cpm) per 20×10^6 sperm.

Total Antioxidant Capacity

TAC was measured in seminal plasma using the enhanced chemiluminescence assay. Aliquots of the seminal plasma stored at -20°C were thawed at room temperature and immediately assessed for their antioxidant capacity as follows. Seminal plasma was diluted 1:10 with deionized water (dH_2O) and filtered through a 0.20- μ m Millipore filter (Allegiance Healthcare Corp., McGaw Park, Ill., USA). Signal reagent was prepared using a chemiluminescence kit (Amersham Life Science, Bucks., UK). 20 μ l of horseradish peroxidase (HRP)-linked immunoglobulin (Amersham Life Science) was added to 4.98 ml dH_2O . This was further diluted 1:1 to give a working solution with the desired luminescence output (3×10^7 cpm). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water-soluble tocopherol analogue, was added as the standard at concentrations between 50 and 150 μM . With the luminometer set in the kinetic mode, 100 μ l of signal reagent and 100 μ l of HRP were added to 700 μ l of dH_2O and mixed. The solution was then equilibrated to the desired level of chemiluminescence output (between 2 and 3×10^7 cpm) for 100 s. 100 μ l of the prepared seminal plasma was added to the signal reagent and HRP, and the chemiluminescence measured. Suppression of chemiluminescence and the time from the addition of seminal plasma to 10% recovery of the initial chemiluminescence was recorded. Antioxidant capacity was expressed as molar Trolox equivalents.

ROS-TAC Score

The ROS and TAC values from the controls were used to create a scale of these two variables that uses the control values as reference points. The log of (ROS + 1) was used in calculations so that both values were normalized to the same distribution. First, both TAC and log (ROS + 1) were standardized to z-scores so that both would have the same variability. These standardized scores were calculated by subtracting the mean values for the controls from the mean value for the patients and dividing by standard deviation of the control population.

For log (ROS + 1): Standardized ROS = $[\log(\text{ROS} + 1) - 1.3885]/0.7271$

For TAC: Standardized TAC = $(\text{TAC} - 1650.93)/532.22$

These two standardized variables were then analyzed with the principal components analysis, which provided linear combinations (or weighted sums) that account for the most variability among correlated variables. The first principal component provided the following linear equation:

Principal component = $(-0.707 \times \text{standardized ROS}) + (0.707 \times \text{standardized TAC})$

To ensure that the distribution of the ROS-TAC score would have a mean of 50 and standard deviation of 10 in controls, the ROS/TAC score was transformed as:

ROS-TAC score = $50 + (\text{principal component} \times 10.629)$

ROS-TAC score was formulated using principal components to predict fertility potential in these men.

Previous reports from our laboratory have demonstrated that the ROS-TAC score is superior to ROS or TAC alone in predicting fertility during follow-up of patients with male-factor infertility [7]. Therefore, we compared patients with idiopathic infertility

with fertile (n = 13) and infertile (n = 39) men with male factor diagnoses, and probability of remaining infertile 1 year after the infertility diagnosis was calculated based on logistic regression estimates of the known fertile and infertile males were calculated. The probabilities of infertility in these patients were examined based solely on the logistic regression estimates. These, in turn, were based on the known fertility status of the patients and were used to examine the potential clinical relevance of the observed ROS-TAC levels.

Semen Score

A principal component analysis model can be employed to calculate overall semen scores that accounts for most of variability observed among the battery of interrelated semen variables.

Based on the fact that semen variables are interrelated, the relationships among the semen parameters and the feasibility of composite scores that represents most of the overall variability of the semen parameters were tested. The semen scores were calculated as follows:

(1) Nine semen parameters identified according to the WHO guidelines were included: concentration, motility, sperm morphology according to WHO guidelines and Tygerberg strict criteria, VCL, VSL, VAP, LIN, and ALH.

(2) Rather than using the raw values, base 10 logarithms of all the variables after adding a value of 1 were used. The reasons for this conversion were to reduce the effect of high outliers, to make the variables more comparable in scale (puts all variable measures in millions and those recorded in percentages on a more level playing field), and to completely reduce the effect of recording variables as 3,106 or 3,104.

(3) Principal component analysis was applied to the covariance matrix of the 9 log-transformed semen parameters. This produced 9 new components, which were weighted sums of the original variables. In matrix algebra nomenclature, the weights are referred to as 'eigenvectors'. We were interested mainly in components that would account for at least 10% of the overall variability of the 9 semen parameters. The use of a log transformation will result in variables with the highest coefficient of variation being assigned higher weights.

(4) Since the correlations among the semen parameters were positive, it was anticipated that most of the variability would be explained by a weighted sum of the whole variables, which would be related to the overall SQ.

(5) The first principal component accounted for 64.8%, or almost two thirds, of the overall variability observed among the 9 variables. Therefore, this first principal component summed up all the semen parameters with varying weights given to each component. This score was referred to as the 'SQ score' to represent the overall SQ.

The SQ score was calculated as follows:

SQ score:

$$\begin{aligned} & (\log \text{concentration} \times 0.807647 + \log \text{motility} \times 0.254114 \\ & + \log \text{strict criteria morphology} \times 0.331037 \\ & + \log \text{WHO morphology} \times 0.274769 + \log \text{VCL} \times 0.146286 \\ & + \log \text{VSL} \times 0.193481 + \log \text{VAP} \times 0.175990 \\ & + \log \text{LIN} \times 0.009109 + \log \text{ALH} \times 0.090307) \\ & \times (10/0.2901900) - 20.1911369 \end{aligned}$$

The second principal component accounted for 15.6% of the variability from the 35.2% not accounted for by the SQ score. This

Table 1. Comparison of semen characteristics, measures of oxidative stress, and semen score between patients with idiopathic infertility and normal controls

Variables	Idiopathic infertility (n = 36)	Controls (n = 19)	p
Concentration, $\times 10^6/\text{ml}$	37.53 \pm 6.89	69.4 \pm 10.03	0.009
Motility, %	37.93 \pm 3.36	55.5 \pm 4.8	0.003
WHO morphology, %	30.39 \pm 2.23	39.8 \pm 3.2	0.02
Semen score	83.0 \pm 14.5	100.0 \pm 10.0	0.001
log (ROS + 1)	2.3 \pm 0.21	1.3 \pm 0.3	0.006
TAC	1,014.75 \pm 79.22	1,653 \pm 115.29	0.001
ROS-TAC score	32.8 \pm 14.2	50.0 \pm 10.0	0.001

All values are expressed as mean \pm SE. * p < 0.05 was considered statistically significant.

second variable was a weighted sum of 8 variables, which subtracts concentration multiplied by the weight. High scores on this scale are related to high motility, good morphology, or high scores on other parameters relative to their concentration. Therefore, this score was 'relative SQ score' or RQ score. The calculation was as follows:

RQ score:

$$\begin{aligned} & (\log \text{concentration} \times -0.567457 + \log \text{motility} \times 0.147947 \\ & + \log \text{strict criteria morphology} \times 0.605335 \\ & + \log \text{WHO morphology} \times 0.418694 \\ & + \log \text{VCL} \times 0.180399 + \log \text{VSL} \times 0.202853 \\ & + \log \text{VAP} \times 0.189159 + \log \text{LIN} \times 0.009502 \\ & + \log \text{ALH} \times 0.069321) \times (10/0.1813407) + 24.5183708 \end{aligned}$$

The two new semen scores, SQ and RQ, together account for more than 80% of the variability observed among the original 9 semen parameters.

Statistical significance was assessed with two-tailed tests at the p < 0.05. Summary statistics are presented as mean \pm SE. Statistical tests were performed using SAS Version 6.12 (SAS Institute, Cary, N.C., USA).

Results

Compared to controls, patients with idiopathic infertility had significantly lower sperm concentration (37.53 \pm 6.89 vs. 69.4 \pm 10.03; p < 0.009), sperm motility (37.93 \pm 3.36 vs. 55.5 \pm 4.8; p < 0.003), and normal morphology (30.39 \pm 2.23 vs. 39.8 \pm 3.2; p < 0.02) (table 1). The idiopathic group had lower semen quality scores (83.0 \pm 14.5) than controls (100.0 \pm 10.0) (p < 0.001).

ROS levels (using the log transformation) were higher in men with idiopathic infertility (2.3 \pm 0.21) compared

Table 2. Comparison of semen characteristics and measures of oxidative stress between proven fertile and infertile patients, and patients with idiopathic infertility

Variables	Study groups			p*		
	fertile (n = 13)	infertile (n = 39)	idiopathic infertility (n = 36)	A ^a	B ^b	C ^c
Concentration, × 10 ⁶ /ml	42.6 ± 13.8	30.3 ± 8.1	37.53 ± 6.89	0.17	0.32	0.51
Motility, %	32.3 ± 5.7	38.4 ± 3.3	37.93 ± 3.36	0.40	0.28	0.94
WHO morphology, %	32.8 ± 3.6	29.8 ± 2.2	30.39 ± 2.23	0.27	0.57	0.71
log (ROS + 1)	1.9 ± 0.4	2.3 ± 0.2	2.3 ± 0.2	0.28	0.26	0.88
TAC	1,862.2 ± 146.4	1,299.9 ± 84.5	1,014.8 ± 57.9	0.02	<0.001	0.03
ROS-TAC score	47.7 ± 4.3	35.8 ± 2.5	32.2 ± 2.7	0.02	0.009	0.35

All values are expressed as mean ± SE. ^a Fertile patients vs. infertile patients; ^b fertile patients vs. idiopathic infertility; ^c infertile patients vs. idiopathic infertility. * p < 0.05 was considered statistically significant.

to controls (1.3 ± 0.3) (p = 0.006), whereas the TAC level was lower in men with idiopathic infertility (1,014.75 ± 79.22) compared to controls (1,653 ± 115.29) (p = 0.001). Idiopathic infertility patients had significantly lower ROS-TAC scores (32.8 ± 14.2) than controls (50.0 ± 10.0) (p < 0.001) (table 1).

An estimated 64% of men with idiopathic infertility will remain infertile during 1-year follow-up based on logistic regression analysis.

Discussion

Standard semen analysis using a light microscope is widely used in most laboratories for the initial evaluation of the male partner of an infertile couple [9, 11]. However, manual analyses can be very subjective and prone to within- and between-observer technical error [32–34]. Any laboratory analysis is subject to two potential sources of error: random (sampling error) and systematic (observer bias) [15]. According to WHO guidelines (1999), a sperm concentration below 20 × 10⁶/ml is abnormal [35]. However, these guidelines are not based on studies of fertility but are arbitrarily defined by a committee of international experts. The so-called normal values provided by the WHO manuals for the basic semen parameters (i.e., volume and qualitative and quantitative motility and morphology) were obtained mostly through studies of the so-called fertile populations. This may be a reason why the clinical value of traditional semen characteristics in the assessment of male infertility is a subject of considerable debate.

In addition, diagnosing defective sperm function by standard semen analysis is difficult because spermatozoa are highly specialized cells that express a diverse array of biological properties to achieve fertilization [8, 9, 11]. In our study, conventional sperm characteristics (sperm concentration, motility, and normal forms) were completely normal in a subset of infertile men diagnosed as idiopathic, but were different from the fertile donors. Thus, the finding of normal conventional sperm parameters does not guarantee that the sperm population in an ejaculate will be fertile. Therefore, biochemical seminal parameters may distinguish a fertile from an infertile man.

There is growing evidence to suggest that seminal oxidative stress is involved in many aspects of male infertility [5–7, 16–23]. Increased levels of seminal oxidative stress have been correlated with sperm dysfunction through different mechanisms that include lipid peroxidation of sperm plasma membrane and impairment of sperm metabolism, motility, and fertilizing capacity [19, 20, 31]. In addition, oxidative stress has been shown to affect the integrity of the sperm chromatin and to cause high frequencies of single and double DNA strand breaks [36–39]. The biological impact of an abnormal sperm chromatin structure depends on the combined effects of the extent of DNA or chromatin damage in the spermatozoa and the capacity of the oocyte to repair that damage [36–42]. Using the comet assay, a study has indicated that ejaculated sperm from infertile men had single- and double-stranded DNA breaks and that both were more prevalent in men with abnormal semen parameters [40]. Another explanation for the link between seminal oxidative

stress and sperm DNA damage may be related to a defect in spermiogenesis that causes the release of spermatozoa that are immature and have abnormal chromatin structure/high DNA damage and abnormal morphology [43]. Spermatozoa with abnormal morphology have been shown to have a capacity to generate high levels of ROS that, on exceeding critical levels, can cause oxidative stress [23, 44].

Previous reports have described that 25–40% of patients with idiopathic infertility have elevated levels of ROS [2]. This would suggest that lipid peroxidation of sperm membrane may be one of the key mechanisms involved in the pathophysiology of male infertility [23]. In our study, ROS levels were higher in men with idiopathic infertility compared to controls, whereas the TAC level was lower in men with idiopathic infertility compared to controls. In addition, idiopathic infertility patients had significantly lower ROS-TAC scores than controls. Of utmost importance, an estimated 64% of men with idiopathic infertility will remain infertile during 1-year follow-up based on logistic regression analysis.

Numerous studies have reported the predictive value of semen parameters such as concentration of motile spermatozoa, quantitative and computerized measurements of spermatozoa motility, morphology, zona pellicula binding, and occurrence of the acrosome reaction to aid in the determination of in vitro fertilization and pregnancy outcome [45–49]. After the introduction of CASA, the number of semen characteristics examined has increased to the extent that each semen evaluation quantifies more than 10 semen characteristics [13]. Although these characteristics are unique measures of semen quality, they are not independent of one another in the sense that patients with low motility tend to have low concentration and vice versa. Therefore, semen characteristics are positively correlated with each other [15].

The introduction of CASA made it possible to measure motility characteristics of individual spermatozoa [13, 14]. CASA is widely accepted as providing a rapid and objective measurement of individual 'classical' sperm characteristics such as sperm count and sperm movement [10]. CASA also increases the accuracy and reproducibility of sperm count and motility and allows the determination of sperm motion characteristics, termed 'kinematics', which cannot be obtained by microscopic observation, and may be important in determining the fertility potential of sperm [13, 14].

Perhaps the most widely utilized semen characteristic is sperm count. Men with less than 20×10^6 spermatozoa/ml are typically deemed subfertile, and men with counts less than 5×10^6 spermatozoa/ml are often considered infertile [15]. Semen samples containing less than 14% normal forms by Tygerberg strict criteria are reported as subfertile, and those containing less than 5% normal forms are considered severely impaired, causing some centers to recommend couples discontinue treatment and move on to donor insemination [48, 50]. However, like its predecessors, strict sperm morphology is not absolutely accurate in predicting fertility. In recent years, deficiencies in using these measures of semen parameters have been reported, and the predictive value of spermatozoa concentration, for example, has been criticized because of the natural day-to-day variations that occur in spermatozoa concentration [15]. The semen score can provide important information on the SQ and the likelihood of establishing a pregnancy [15]. Also, semen scores provide more meaningful information than the individual semen characteristics. Patient scores <80 are below the expected normal range of donors. In our study, the SQ score was 83.0 ± 14.5 .

Conclusions

In our study we can therefore conclude that patients with idiopathic infertility have lower scores of semen quality and markers of oxidative stress (ROS, TAC, and ROS-TAC score) compared to controls. 64% of men with idiopathic infertility tend to remain infertile within 1 year. Both semen and ROS-TAC scores provide important information about the semen quality and fertilizing potential; this information may be used on the medical management of infertile patients with idiopathic etiologies.

Acknowledgment

The authors thank Dave Nelson who performed all the statistical analysis.

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