# Preoperative Seminal Plasma Disturbed Oxidant/Antioxidant Milieu Could Predict Failure of Varicocelectomy as a Therapeutic Modality for Male Infertility

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**Abstract:** Objectives: To evaluate seminal plasma (SP) malonaldehyde (MDA), superoxide desmutase (SOD) and CoQ10 levels in infertile men with and without varicocele as a trial to evaluate the predictability of their preoperative estimation for postoperative (PO) improvement of seminal parameters. Patients & Methods: 70 infertile men; with (n=35) and without (n=35) and 20 fertile men with (n=10) and without (n=10) clinically and ultrasonographically diagnosed varicocele. Infertile men with varicocele were assigned to undergo bilateral varicocelectomy using subinguinal approach. All infertile patients had preoperative hormonal profile. Semen samples were obtained from all men for standard semen analysis and SP estimation of CoQ10 using high-performance liquid chromatography (HPLC), SP MDA using a thiobarbituric acid reactive substances (TBARS) assay and SP SOD activity by chemiluminescence. Another semen sample was obtained PO for standard semen analysis. Results: Baseline data showed a significant decrease of sperm concentration and percentage of sperms with rapid progressive motility with significantly higher percentage of sperms with abnormal morphology and significantly lower SP CoQ10 and SOD levels with significantly higher SP MDA levels in infertile men compared to fertile men with significant difference among infertile men in favor of those free of varicocele. In all infertile men, there was negative significant correlation between SP MDA and CoQ10 levels and a positive significant correlation between SP SOD and CoQ10 levels with higher significance in those had varicocele. In infertile men free of varicocele, there was a negative correlation between sperm count and SP MDA with a positive significant correlation with SP SOD levels, while in infertile men with varicocele, the correlation was significant between SP SOD and the three seminal parameters and between the percentage of abnormal sperm forms and SP MDA and CoQ10. PO sperm count showed a negative significant correlation with preoperative SP MDA levels, while the correlation was positive significant with SP CoQ10 and SP SOD levels. Regression analysis identified high preoperative SP SOD level as significant predictor of improvement of sperm count after varicocelectomy. ROC curve analysis defined preoperative SOD level at ≥88 U/ml as a specific predictor for PO improvement of sperm count, while identified preoperative SP MDA level at ≥0.53 nmol/ml and SP CoQ10 at cutoff point of 0.12 µg/ml could identify infertile men with varicocele most probably will not get benefit of varicocelectomy. Conclusion: Combined varicocele and disturbed oxidant/antioxidant system could be the underlying mechanism for varicocele associated male infertility and highly disturbed oxidant/antioxidant system could influence the outcome of varicocelectomy as a therapeutic modality. Preoperative estimation of SP levels of MDA and SOD could aid to predict the outcome of varicocelectomy.

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## 1. Introduction:

Oxidative stress is believed to underlie the etiology of numerous human conditions. Organisms are subject to oxidative stress from endogenous and exogenous sources which can induce severe macromolecular, cellular and tissue damage through direct cytotoxic effects, promotion of primary genotoxic events, or generation of reactive oxygen intermediates such as the superoxide anion and hydroxyl radical, [(1)].

Reactive oxygen species (ROS) can be produced by human spermatozoa and as a result of high polyunsaturated fatty acid content, human spermatozoa plasma membranes are highly sensitive to ROS-induced damage and hydrogen peroxide appears to be the most toxic ROS for human spermatozoa. There is growing evidence that peroxidative damage to the human spermatozoa membrane is an important pathophysiological mechanism in human male infertility, [(2, 3)].

Human spermatozoa and seminal plasma possess various antioxidant systems to scavenge ROS and prevent ROS-related cellular damage. Failure of antioxidant defenses to detoxify excess ROS production can lead to significant oxidative damage including enzyme inactivation, protein degradation

and lipid peroxidation. Mitochondrial DNA is believed to be both source and target of ROS, and unlike nuclear DNA is not compactly packed and hence more susceptible to oxidative stress than nuclear DNA. Antioxidant defense systems, which are involved in a variety of detoxification reactions, exhibit baseline levels of activity to ensure the maintenance of the balance between production and removal of endogenous ROS and other pro-oxidants, [4, 5].

Varicocele is one of the leading causes of male infertility, and is present in almost 40% of infertile males. Understanding the role of oxidative stress in male reproduction has led some researchers to postulate oxidative stress as the possible cause of sperm dysfunction in varicocele patients, [6]. The objective of the present study was to evaluate the seminal plasma levels of MDA, SOD and CoQ10 as a sample of oxidant/antioxidant system in infertile men with and without varicocele and to conduct a trial to evaluate the predictability of their preoperative estimation for postoperative improvement of seminal parameters.

**2. Patients and Methods:**

This prospective selective comparative study was conducted at Clinical Pathology & General Surgery Departments, Benha University & Zagazig University Hospitals and was designed to comprise 35 infertile men with varied degrees of varicocele, 35 infertile men free of varicocele both clinically and by ultrasonographic examination and 20 age-matched fertile men; 10 had varicocele and 10 free of varicocele. All infertile patients underwent preoperative complete hormonal profile evaluation to exclude associated endocrinopathy.

All men were evaluated clinically in standing position with and without Valsalva maneuver for clinical detection and grading of varicocele, if present, as grade 1 (palpable with Valsalva), grade 2 (palpable without Valsalva), and grade 3 (visible through the scrotal skin). Ultrasonographic examination was conducted for assurance of varicocele grade, measurement of testicular dimensions, and testicular volume (in $cm^3$) was calculated by multiplying (0.53 length, width, depth). A testicular volume of <19 $cm^3$ was considered hypotrophic and a testicular volume difference between the right and left testicles of ≥3 $cm^3$ was considered asymmetric, [7, 8]. Infertile men with varicocele were assigned to undergo bilateral varicocelectomy using subinguinal approach.

Laboratory investigations
Sampling:

Semen samples were obtained from all men following a minimum of 3 days and a maximum of 5 days sexual abstinence. Immediately after sample donation, about 0.5 ml of the collected sample was obtained and stored in dark to protect against photodegradation of ubiquinone and frozen at -20°C till be assayed SP CoQ10 level. The remaining sample was allowed for liquefaction and divided into two parts: the first for a routine semen analysis, but the second part was centrifuged for 7 minutes and seminal plasma was removed and stored at -80°C till be assayed. Another semen samples were obtained PO from infertile men with varicocele for routine semen analysis.

Investigations:

Standard semen analysis, using light microscopy, was performed according to World Health Organization criteria [9] to determine sperm concentration, motility and morphology. Sperm motility was expressed as the percentage of sperms showed rapid progressive forward motility (those spermatozoa which exhibits actual space-gain motility). Morphological assessment was performed at x100 magnification under oil-immersion and results were expressed as the percentage of abnormal spermatozoa observed on each slide; normal spermatozoa is that has a smooth, oval configuration with a well-defined acrosome incorporating 40–70% of the sperm head, no neck, midpiece or tail defects, and no cytoplasmic droplets more than one-half the size of the sperm head.

CoQ10 was assayed by high-performance liquid chromatography (HPLC), employing a UV detector (275 nm), according to Littarru et al., [10]. Seminal plasma, 0.5 ml supplemented with 2 ml of ethanol:isopropanol (95:5), 0.5 ml of 0.1 M sodium dodecyl sulfate and 0.5 µg of coenzyme Q8 (CoQ8) as an internal standard, was extracted twice with 4 ml of n-hexane. Combined extracts were brought to dryness under nitrogen and re-dissolved in 100 µl of ethanol. An aliquot of 20 µl was injected into the HPLC apparatus, whose conditions were as follows: column, ultrasphere octodecylsilane, 250 x 4.6 mm; mobile phase, ethanol-methanol (70:30); detector, UV 275 nm. The levels were expressed as concentrations (µg/ml).

Malonaldehyde (MDA) measurements: lipid peroxide levels in the seminal plasma was measured using a thiobarbituric acid reactive substances (TBARS) assay, which monitors MDA production based on the method of Beuge & Aust [11]. Briefly, to 100 µl sample of seminal plasma, 200 µl of cold 1.15% (w/v) KCl was added to 1.8 ml of 3% phosphoric acid and 0.6 ml of 0.6% TBA. These mixtures were heated in boiling water for 45 min.

After cooling, the MDA was extracted by centrifugation at 1,500 × g for 10 min at 25ºC and the intensity was measured at 535 nm using ultraviolet-visible spectrophotometry. The MDA level was determined using the molar absorption coefficient of the MDA at 535 nm 1.56×105 mol/L/cm.

Superoxide dismutase activity was quantified by chemiluminescence [12] using the xanthine/XO lucigenin assay. A final volume of 1 ml contained the following components: 100 µl seminal plasma, 100 mM diethylenetriaminepentaacetic acid (DTPA); 100 mM lucigenin, 180nM XO, and 50 mM xanthine in 50 mM HEPES, pH 7.4. The reaction was started by adding xanthine, and the resulting photon emission was recorded in Bertold LB 9505 C luminometer at 25ºC. Bovine CuZn SOD was used for calibration. One unit represents the concentration of SOD required to inhibit the release of superoxide by 50% and equals 5 nM Cu.

Statistical analysis

Obtained data were presented as mean±SD, ranges, numbers and ratios. Results were analyzed using Wilcoxon Signed Ranks Test for comparisons between groups and Chi-square ($X^2$-test) for numbers and percentage comparisons. Possible relationships were investigated using Pearson's linear correlation coefficient and Regression analysis (Stepwise method) was used for identification of predictors for PO sperm count improvement. ROC curve analysis, judged by area under curve (AUC), was used for evaluation of cutoff points of each parameter as predictor. Statistical analysis was conducted using the SPSS (Version 10, 2002) for Windows statistical package. P value <0.05 was considered statistically significant.

**3. Results:**

There was non-significant (p>0.05) difference between studied groups as regards to age that ranged between 23.5 and 39.5; mean: 30.6±2.7 years. Infertile group comprised 70 patients had a mean duration of infertility of 3.6±1.4; range: 1-6 years with a non-significant (p>0.05) difference between both infertility subgroups. Twenty-three testicles in infertile group were hypotrophic, while only 4 testicles were hypotrophic in fertile group, with a significantly ($X^2$=4.356, p<0.01) higher frequency of hypotrophic testicles in the infertile patients and non-significantly higher frequency among testes free of varicocele compared to those had varicocele, (Table 1). Among infertile patients with varicocele, 15 patients had bilateral varicocele of grade III, 12 patients had bilateral varicocele of grade II and 8 patients had bilateral varicocele of grade I, while among fertile men with varicocele, 5 patients had bilateral varicocele of grade I, 3 patients had bilateral varicocele of grade II and only 2 patients had bilateral varicocele of grade III.

Baseline seminal parameters showed a significant (p<0.05) decrease of sperm concentration and percentage of sperms with rapid progressive motility with significantly higher percentage of sperms with abnormal morphology in infertile compared to fertile men and in those had varicocele compared to those free of varicocele. Varicocelectomy significantly improved sperm concentration and percentage of motile sperms and significantly reduced the percentage of sperms with abnormal forms (Table 2).

Baseline SP CoQ10 and SOD levels were significantly lower and SP MDA levels were significantly higher in infertile men compared to fertile men with significant difference between infertile men in favor of those free of varicocele (Table 3).

In all infertile men, there was negative significant correlation between SP MDA and CoQ10 levels and a positive significant correlation between SP SOD and CoQ10 levels with higher significance in those had varicocele. Moreover, there was a negative correlation between SP SOD and MDA in infertile men and was significant in those had varicocele (Table 4).

The impact of disturbed testicular oxidative milieu on sperm quality was manifested as negative significant correlation between sperm count in infertile men without varicocele and SP MDA with a positive significant correlation with SP SOD levels. Such impact was intensified by the presence of varicocele in infertile men with varicocele, where the correlation between SP SOD was significant with the three seminal parameters and correlation between the percentage of abnormal sperm forms and SP MDA and CoQ10 became significant (Table 4).

Moreover, in infertile patients with varicocele, PO sperm count showed a negative significant correlation with preoperative SP MDA levels, (r=-0.4, p=0.029), while the correlation was positive significant, (r=0.432 & 0.498, p=0.002, respectively) with SP CoQ10 and SP SOD levels (Table 4, Fig. 1).

Regression analysis of preoperatively estimated SP parameters identified high SP SOD level as significant predictor, in 2 models, of improvement of sperm count after varicocelectomy, while high preoperative SP MDA level was significant in only one model and its exclusion minimal affected significance of preoperative SP SOD as a predictor (Table 5).

For cutoff point verification, ROC curve analysis defined preoperative SOD level at ≥88 U/ml

as a specific predictor for postoperative improvement of sperm count with AUC=0.621, (Fig. 2), while preoperative SP MDA level at ≥0.53 nmol/ml could identify infertile men with varicocele most probably will not get benefit of varicocelectomy with AUC=0.823 (Fig. 3). On the other hand, preoperative SP CoQ10 level could be used as screening test with high sensitivity for the prediction of varicocelectomy failure at cutoff point of >0.12 µg/ml with AUC=0.270 (Fig. 4).

**Table (1): Testicular dimension data**

| | | | Testicular size | | Testicular symmetry | |
|---|---|---|---|---|---|---|
| | | | Mean±SD ($cm^3$) | Number of hypo-trophic testes | Testicular size difference ($cm^3$) | Number of asymmetric |
| Fertile men | Free of varicocele | Rt | 26.3±2.4 | 1 | 2.43±0.6 | 4 |
| | | Lt | 23.8±2.8 | 2 | | |
| | varicocele | Rt | 23.7±2.1 | 0 | 2.44±1 | 1 |
| | | Lt | 21.2±2.2*† | 1 | | |
| Infertile men | Free of varicocele | Rt | 21.6±4.9† | 6 | 2.12±0.9 | 4 |
| | | Lt | 21.1±5.5† | 9 | | |
| | varicocele | Rt | 23±2 | 3 | 2.45±1.17 | 5 |
| | | Lt | 20.9±2.1*† | 5 | | |

Data are presented as means±SD & numbers
Hypotrophic testis means testicular size <19 $cm^3$
Asymmetric testes means testicular size difference >3 $cm^3$
*: significant versus right testicular size.
†: significant versus fertile men free of varicocele.

**Table (2): Seminal analysis data recorded in studied groups**

| Group / Parameter | Fertile Men | | Infertile Men | | |
|---|---|---|---|---|---|
| | Free of varicocele | With varicocele | Free of varicocele | With varicocele | |
| | | | | Preoperative | Postoperative |
| Count ($10^6$/ml) | 67.1±13.6 | 39.9±15.7 | 28.3±7.5 | 20.5±5.3 | 24.1±6.3 |
| | Z=2.805, $p_1$=0.005 | | Z=5.627, $p_1$<0.001 | | Z=5.221, $p_4$<0.001 |
| | | | Z=7.156, $p_2$<0.001 | Z=4.763, $p_3$<0.001 | |
| Motility (%) | 72.9±11.3 | 64±8.2 | 48.7±8.5 | 34.1±5.9 | 54.1±9.4 |
| | Z=1.788, $p_1$>0.05 | | Z=5.328, $p_1$<0.001 | | Z=5.163, $p_4$<0.001 |
| | | | Z=6.354, $p_2$<0.001 | Z=4.154, $p_3$<0.001 | |
| Abnormal forms (%) | 13.8±4.2 | 22.2±4.3 | 25.9±8.1 | 34.2±10.6 | 23.9±7.4 |
| | Z=7.156, $p_1$<0.001 | | Z=4.763, $p_1$<0.001 | | Z=5.162, $p_4$<0.001 |
| | | | Z=7.156, $p_2$<0.001 | Z=4.763, $p_3$<0.001 | |

$p_1$: significance versus men free of varicocele irrespective of fertility
$p_2$: significance versus fertile men with varicocele $p_3$: significance versus fertile men free of varicocele
$p_4$: significance versus preoperative measures

**Table (3): Baseline SP MDA, CoQ10 and SOD levels estimated in studied groups**

| | Fertile | | Infertile | |
|---|---|---|---|---|
| | With varicocele | Free of varicocele | With varicocele | Free of varicocele |
| MDA (nmol/ml) | 0.34±0.03 | 0.45±0.046 | 0.54±0.08 | 0.48±0.07 |
| Statistical analysis | Z=2.713, $p_1$=0.007 | | Z=2.807, $p_1$=0.005 | |
| | | | Z=3.156, $p_2$=0.001 | Z=1.432, $p_3$>0.05 |
| CoQ10 (µg/ml) | 0.25±0.032 | 0.2±0.043 | 0.11±0.031 | 0.16±0.035 |
| Statistical analysis | Z=2.397, $p_1$=0.017 | | Z=4.624, $p_1$<0.001 | |
| | | | Z=5.392, $p_2$<0.001 | Z=2.245, $p_3$=0.025 |
| SOD (U/ml) | 106.3±11 | 121.3±8.1 | 87.4±10 | 96.5±8.4 |
| Statistical analysis | Z=2.346, $p_1$=0.019 | | Z=3.027, $p_1$=0.002 | |
| | | | Z=7.576, $p_2$<0.001 | Z=8.468, $p_3$<0.001 |

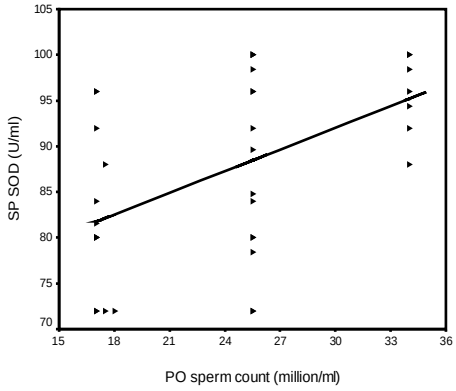
$p_1$: significance versus men free of varicocele irrespective of fertility
$p_2$: significance versus fertile men with varicocele $p_3$: significance versus fertile men free of varicocele.

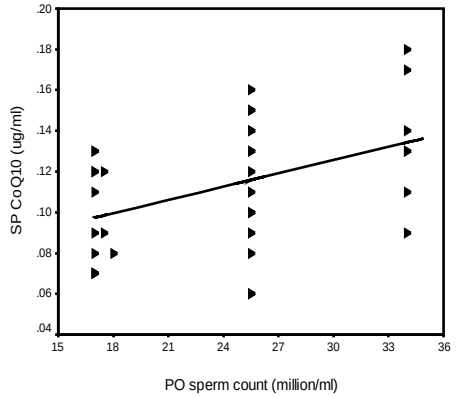
**Table (4): Correlation coefficient between estimated seminal parameters in infertile men and with PO sperm count in infertile patients with varicocele**

| | Infertile with varicocele | | | | | | Infertile free of Varicocele | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | SP MDA | | SP CoQ10 | | SP SOD | | SP MDA | | SP CoQ10 | | SP SOD | |
| | r | p | r | p | r | p | r | p | R | p | r | p |
| Sperm count | -0.14 | >0.05 | 0.312 | >0.05 | 0.541 | =0.001 | -0.44 | 0.008 | 0.238 | >0.05 | 0.489 | =0.003 |
| Sperm motility | -0.15 | >0.05 | 0.283 | >0.05 | 0.444 | =0.007 | -0.13 | >0.05 | 0.254 | >0.05 | 0.169 | >0.05 |
| Abnormal forms | 0.393 | =0.035 | -0.512 | =0.002 | -0.42 | =0.011 | 0.234 | >0.05 | -0.11 | >0.05 | -0.3 | >0.05 |
| SP MDA | | | -0.39 | =0.022 | -0.54 | =0.001 | | | -0.37 | =0.042 | -0.16 | >0.05 |
| SP CoQ10 | | | | | 0.523 | =0.001 | | | | | 0.44 | =0.008 |
| PO sperm count | -0.4 | =0.029 | 0.432 | =0.002 | 0.498 | =0.002 | | | | | | |

**Table (5): Regression analysis of estimated seminal plasma parameters for the predictability of PO improvement**

| Model | Parameter | Standardized coefficient | t | Significance |
|---|---|---|---|---|
| Model 1 | SP SOD | 0.498 | 3.302 | =0.002 |
| Model 2 | SP SOD | 0.710 | 4.210 | <0.001 |
| | SP MDA | 0.391 | 2.318 | =0.027 |



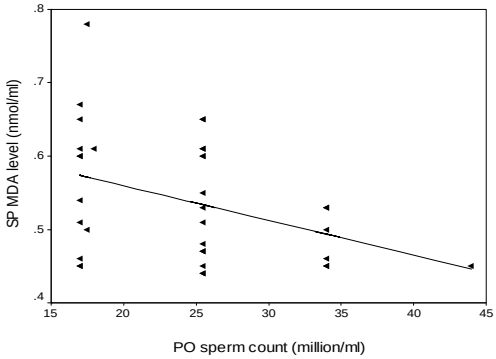




**Fig. (1): Correlation between PO sperm count and preoperatively estimated SP SOD, CoQ10 and MDA levels in infertile patients with varicocele.**

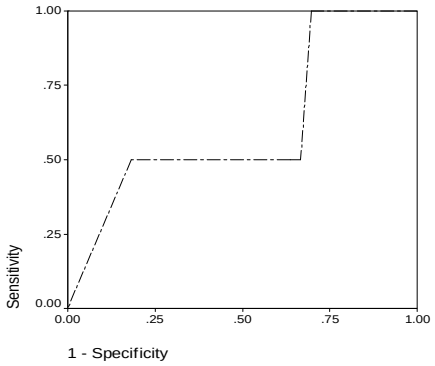



**Fig. (2): ROC curve analysis of preoperative seminal plasma SOD level at cutoff point of ≥88 U/ml as a predictor for postoperative improved seminal characters**

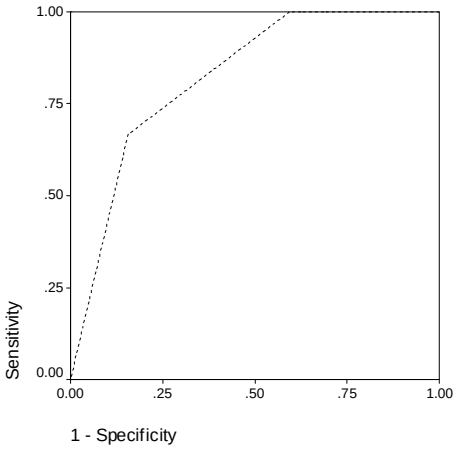



**Fig. (3): ROC curve analysis of preoperative seminal plasma MDA level at cutoff point of ≤0.53 ng/ml as a predictor for postoperative improved sperm count**

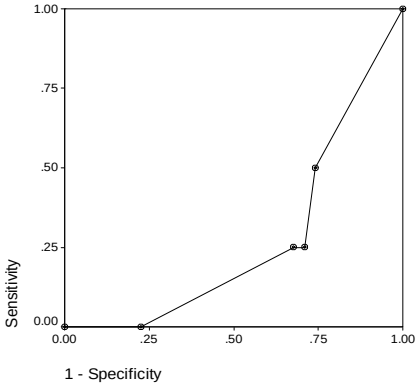



**Fig. (4): ROC curve analysis of preoperative seminal plasma CoQ10 level at cutoff point of 0.12 µg/ml as a predictor for postoperative improved sperm count**

**4. Discussion:**

The current study reported significant improvement of seminal parameters after varicocelectomy using the subinguinal approach with special regard to the percentage of progressive forward motile sperms. These data point to the beneficial effect of varicocelectomy on the fertility status of infertile couple due to a male factor and go in hand with Ficarra et al. [13] who found varicocele repair has a beneficial effect on fertility status in infertile men with palpable varicocele with a significant increase in pregnancy rate in patients who underwent varicocele treatment (36.4%) compared with patients having no treatment (20%). Moreover, French et al. [14] reported that with regard to the efficacy of varicocele repair, previous meta-analysis of the available data has been misleading due to improper selection criteria and the findings of the Cochrane database review, a study that has been accepted by many as evidence against varicocele repair; however, varicocele repair was found not only as an effective treatment for appropriately selected patients but can also be the most cost effective option.

The impact of disturbed oxidant/antioxidant milieu on testicular function and spermatic capacitation was evident and manifested as higher levels of SP MDA and lower SP CoQ10 and SOD in infertile men free of varicocele compared to fertile men free of varicocele. These data go in hand with multiple studies previously evaluated the impact of disturbed oxidant milieu on testicular functions; Aydemir et al. [15] suggested that Cu and Fe might be mediators of the effects of oxidative damage on spermatogenesis and male infertility and the determination of Fe and Cu levels in serum and seminal plasma during infertility investigation is recommended. Li et al. [16] reported significant difference in SP CoQ10 concentrations between fertile and infertile men. Aydemir et al. [17] suggested that the susceptibility of sperm and seminal plasma to oxidative stress is significantly greater in idiopathic infertile men with the glutathione S-transferase Mu-1 null genotype compared with those possessing the gene and therefore, in patients with idiopathic infertility, GSTM1 polymorphism might be an important source of variation in susceptibility of spermatozoa to oxidative damage.

The risk of oxidative damage was intensified by the presence of varicocele as manifested by significantly higher SP MDA with lower SP SOD and CoQ10 in infertile men had varicocele compared to those free of varicocele. In support of this finding, there was a positive significant correlation between seminal parameters and SP SOD and CoQ10 and negative with SP MDA; a finding indicating the deleterious effect of presence of varicocele on the disturbed oxidant/antioxidant milieu with its harmful effect on testicular function. Also, the reported positive significant correlation between PO improvement of sperm count and preoperative SP SOD and negative significant correlation with SP MDA level indicated the effect-outcome relationship between preoperative antioxidant capacity and on coming improvement.

These data were further supported statistically where preoperative SP SOD and MDA levels were found as predictor for postoperative change of sperm count and their high levels are specific predictors for outcome, improvement or failure, respectively. These data go in hand with Giulini et al. [18] who evaluated total antioxidant capacity in the seminal plasma of infertile patients with varicocele in relation to their semen parameters

and found seminal plasma total antioxidant capacity concentrations were significantly lower than in controls and normozoospermic patients with varicocele and in patients with severe oligosthenozoospermia than in asthenozoozpermic patients with varicocele and in all subjects, concentrations of TAC showed a positive correlation with sperm concentration and motility.

Also, Abd-Elmoaty et al. [19] reported significantly higher levels of oxidants (malonaldehyde and nitric oxide) and reduced levels of antioxidants (superoxide dismutase, glutathione peroxidase, catalase, and ascorbic acid) are seen in semen of infertile men with varicocele and seminal oxidative stress seen in men with varicocele is associated with sperm motility and grade of varicocele.

In hand with the applicability of estimation of seminal plasma prior to initiation of treatment could predict the treatment outcome, Vankatesh et al. [20], (2009) recommended measurement of seminal reactive oxygen species levels in infertile men for better understanding of the aetiology and selection of antioxidant regimen in the treatment of male infertility. Moreover, Ozbek et al. [21] found a statistically significant difference between mean preoperative and postoperative seminal NO levels, whereas there was no significant difference between mean postoperative seminal NO levels and that of control group and concluded that preoperative increased level of seminal NO levels may play a role in the sperm dysfunction in infertile patients with varicocele and its persistent level may explain failure to achieve fertility.

However, the current study found the highest diagnostic yield for improvement of sperm count will be associated with SP SOD level ≥88 U/ml and SP MDA level ≤0.53 nmol/ml, to our knowledge this is the first study tried to define cutoff points for these SP parameters for defining the surgical outcome of varicocelectomy

In conclusion, combined varicocele and disturbed oxidant/antioxidant system could be the underlying mechanism for varicocele associated male infertility and highly disturbed oxidant/antioxidant system could influence the outcome of varicocelectomy as a therapeutic modality. Moreover, preoperative estimation of SP levels of MDA and SOD could aid to predict the outcome of varicocelectomy. However, proposed cutoff points need further larger scale studies for confirmation.

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