

CORRELATING SPERM REACTIVE OXYGEN SPECIES PRODUCTION AND ITS MORPHOLOGICAL DEFECTS – WHICH CAN BE THE BEST POSSIBLE MORPHOLOGICAL PREDICTOR OF OXIDATIVE DAMAGE IN ROUTINE SCREENING?

Dinesh Kumar. V ^{*1}, Swetasmita Mishra ², Rima Dada ², Shaik Hussain Saheb ³.

^{*1} Assistant Professor, Department of Anatomy, Pondicherry Institute of Medical Sciences, Puducherry, India.

² Laboratory of molecular reproduction and genetics, All India Institute of Medical Sciences, New Delhi, India.

³ Assistant professor, Department of Anatomy, JJM Medical College, Davangere, Karnataka. India.

ABSTRACT

Introduction: Male infertility contributes to nearly 30% of the total infertile population worldwide. Despite the advances in diagnostic workup of an infertile male in majority (40-50%) of the cases aetiology is not defined. Supra-physiological levels of ROS impair sperm function by damaging polyunsaturated fatty acid-rich sperm membrane and mitochondrial and nuclear DNA.

Materials and Methods: In this study, we performed Chemiluminescence based estimation of Reactive Oxygen Species levels (ROS) in 40 men with idiopathic infertility and 40 age-matched fertile men who served as controls. We had correlated the ROS levels with morphological defects to find out the single best predictor for oxidative stress in sperm.

Results and Discussion: In 74.48% infertile men, seminal ROS levels were higher than the critical value ($d'' 22$ RLU/sec/million). A significant ($p < 0.05$) difference was observed in the number of head defects, mid-piece defects, cytoplasmic droplets and sperm deformity index (SDI) between cases and controls.

Conclusion: Supra-physiological ROS levels can impair the sperm function, thus resulting in infertility and excessive ROS generation is mostly by the morphologically abnormal spermatozoa. SDI had significant positive correlation with the ROS levels both in cases ($p = 0.00$) and controls ($p = 0.001$) suggesting it as the best surrogate marker for ROS mediated sperm damage.

KEY WORDS: Sperm Reactive Oxygen Species, Male Infertility, Morphological Defects, Oxidative Damage, Chemiluminescence

Address for Correspondence: Dr. Dinesh Kumar. V, Assistant Professor, Department of Anatomy, Pondicherry Institute of Medical Sciences, Puducherry, India. **E-Mail:** dinesh.88560@gmail.com

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INTRODUCTION

A report from the Bertarelli Foundation's second global conference on infertility in the third millennium put it well: "The current treat-

ment of male infertility has become so dominated by the breakthrough technology of ICSI that a kind of nihilism has become widespread in the field." This cynical viewpoint was summed up as follows: "As long as a few motile sperm

are present, no further review of the male is needed" [1].

Recent studies suggest that markers of sperm oxidative stress and DNA integrity may be better measures of male fertility potential than conventional measures [2,3]. The study of sperm DNA damage is particularly relevant in era where assisted reproductive technologies are frequently used which bypass several natural selection steps. Fertilization involves the direct interaction of the sperm and oocyte, fusion of the cell membranes and union of male and female gamete [4]. The completion of this process and subsequent embryo development depend in part on the inherent integrity of the sperm DNA [5].

Till date evaluation of male factors in idiopathic infertility cases, only involves conventional semen analysis and the primitive functional tests. Recent lifestyle factors (exposure to electromagnetic radiations, over usage of cell phone, smoking, excessive consumption of alcohol and increased intake of junk foods) have increased the incidence of oxidative stress and thereby increasing the incidence of male infertility. The supra-physiological levels of reactive oxygen species (ROS) in the male reproductive tract has become a real concern because of their potential toxic effects at high levels on sperm quality and function. ROS are highly reactive oxidizing agents, belonging to the class of free radicals [6]. An oxidative stress test can accurately discriminate between fertile and infertile men and identify patients with a clinical diagnosis of male-factor infertility who are likely to initiate a pregnancy if they are followed over a period of time [7]. In addition, the test can help select subgroups of patients with infertility in which oxidative stress is a significant factor, and who may benefit from antioxidant supplementation. Incorporation of such a test into routine Andrology laboratory practice may be of particular importance to the future management of male infertility [7].

Infertility treatment studies by *Ollero et al.*, [8] have shown that levels of ROS production in semen were negatively correlated with the percentage of normal sperm forms as determined by World Health Organization [9] classification. It has also been proposed that the spermatozoa

itself, in addition to the leukocytes can generate excess ROS especially if it is 'morphologically' abnormal and this ROS can damage the spermatozoa further. Since spermatogenesis is a complex process involving various stages in the formation of mature spermatozoa, disruption at any stage would result in morphologically abnormal spermatozoa, which have been associated with fertilization failure, poor embryo cleavage and increased rate of abortions [10,11].

Though studies have been reported an association between increased ROS production and overall abnormal sperm morphology [12,13] the role of specific inter-morphologic defects in oxidative stress is still to be established. With the advent of techniques like Intracytoplasmic injection of morphologically selected spermatozoa (IMSI), there is a need to group the spermatozoa into different subpopulations.

Wilding M et al., [14] had demonstrated that analysis and selection of spermatozoa for IMSI can improve results in ART cycles through an increase in the number of grade A embryos formed and a decrease in the level of fragmentation in the embryos. Abnormal sperm morphology can be an indicator of one or more defects including damaged DNA, chromosomal abnormalities and centriole deficiency (15, 16). Thus the morphological defects could also serve as a cost-effective first line investigation to delineate patients with oxidative stress and those with DNA damage.

Sperm Deformity Index (SDI) is the score of number of defective spermatozoa to the total number of spermatozoa counted. It is a novel quantitative expression of sperm morphological quality, and is a more powerful predictor of sperm function and the outcome of oocyte fertilization in vitro than either the normal morphology or multiple anomalies index [17]. This study aims at measuring the seminal ROS levels in men with idiopathic male infertility and correlate with morphological defects in spermatozoa.

MATERIALS AND METHODS

In this study, we investigated 40 men with primary infertility, referred from the Urology and Obstetrics and Gynaecology department of All India Institute of Medical Sciences (AIIMS) and

40 fertile controls (fathered a child in last 12 months). The known female factors for Infertility were ruled out after routine investigations. The ethical clearance was obtained prior to the study from the ethical committee, AIIMS (IESC/T-283/01-07-2011) and the cases and controls were enrolled after informed ethical consent.

All cases with constitutional cytogenetic abnormalities and cases with recent (<3 months) history of drug intake, infections and injury were excluded from the study. None of the patient had taken oral antioxidant supplementation or folic acid and none of these cases had history of alcohol intake or smoking. Semen samples were collected from all cases with non-obstructive azoospermia or oligozoospermia with a normal karyotype.

Semen Analysis: Semen samples were collected in a non-toxic, sterile container after sexual abstinence of 72 – 96 hr. They were then evaluated according to World Health Organization Guidelines [9]. The parameters evaluated were pH, semen volume (ml), sperm concentration (million per ml), total sperm count (million) and progressive motility.

Evaluation of Morphological parameters: Ten μ l of the sample was smeared in the glass slide and air dried. The air dried smears were fixed in 90% ethanol for 30 minutes. The fixed smears were stained by haematoxylin and eosin and mounted in DPX. The structure of head, mid piece and tail of the spermatazoa were evaluated under 40X objective. A minimum of 100 spermatozoa were screened for morphological abnormalities of head, mid-piece, tail and other abnormalities. A multiple entry scoring technique was adopted in which an abnormal sperm was classified more than once if more than one deformity was observed.

The Sperm Deformity Index (SDI) [no. of sperm with defects/ total number of spermatozoa counted] and Teratozoospermic Index (TZI) [no. of defects/no. of defective spermatozoa] was calculated for all the cases.

Estimation of Reactive Oxygen Species levels by Chemiluminescence assay in neat semen: The ROS production in 400 μ l of liquefied neat semen was measured after addition of 10 μ l of 5 mM solution of luminol in DMSO (dimethyl

sulphoxide, Sigma Chemical Co.). A tube containing 10 μ l of 5 mM luminol (5-amino-2,3-dihydro-1,4-phthalazinedione, Sigma Chemical Co., St. Louis, MO, USA) solution in DMSO was used as a blank. Chemiluminescence was measured for 10 min using the Berthold detection luminometer (USA). Luminol is an extremely sensitive oxidizable substrate that has the capacity to react with a variety of ROS at neutral pH. The reaction of luminol with ROS results in production of a light signal that is then converted to electrical signal (photon) by a luminometer. Results were expressed in relative light units (RLU) per minute and per million spermatozoa. The RLU/min was then recalculated according to the original spermatozoa concentration in semen sample and expressed as RLU/sec/ million spermatozoa. The test was done using blank (PBS), negative control (PBS + luminol), positive control (PBS+H₂O₂+luminol).

Statistical Analysis: Descriptive statistics such as mean, standard deviation (S.D.), minimum and maximum were computed for all study parameters separately for each group (cases and controls).

To see the significant difference in the study parameters between cases and controls Student-'t' independent test (parametric) was used. Bi-variate correlation and scatter diagram was carried out between ROS levels and other study parameters for cases and controls.

Multiple linear regression analysis was carried out taking ROS as dependent variable and other study parameters as independent variables.

All these statistical analysis was carried out using IBM SPSS software 19.0 version. For all the statistical tests, the level of significance 'P' < 0.05 was considered.

RESULTS

Forty men with idiopathic male infertility and 40 fertile men (who had fathered a child in last one year and had normal sperm parameters) were enrolled as cases and controls. The mean age of the cases was 32.55 \pm 4.82 years and that of the controls was 30.25 \pm 2.34 years. All female factors of infertility were excluded. The infertile men in this study were sporadic cases with no apparent family history of infertility. Infertile men and controls had normal developmental

milestones, no history of chronic obstructive pulmonary disease, infections of male accessory glands, pathologies of inguinal canal or scrotum. None of the study subjects had an occupational exposure to heat, radiation, chemicals and toxins or a recent history (<3 months) of infection or drug intake.

Semen Analysis: Of forty infertile men, 9 cases (22.5%) were oligozoospermic, 7 cases (17.5%) were asthenozoospermic, 7 cases (17.5%) were oligoasthenozoospermic, 4 cases (10%) were azoospermic and the remaining 13 cases (32.5%) had normal sperm parameters.

There was no significant difference in the seminal volume, pH, liquefaction time and viscosity between cases and controls.

Table 1: Comparison of semen parameters in cases and controls values are expressed as mean ± SD.

Category (number)	Ph	Volume (ml)	Liquefaction time (min)	Progressive motility	S.C. (million/ml)
Case (n=40)	7.81±.275	3.25±1.18	32.5±4.40	29.65±19.007	32.05±12.65
Control (n=40)	7.78±.243	3.9±.80	30.5±4.75	53.25±13.981	114.5±44.8

(S.C. -Sperm count, Progressive motility -grade A +B)

Table 2: % of progressive motility (grade A+B), sperm count of subgroups of cases and controls.

Infertile men with sperm pathologies	% of progressive Motility (A+B)	S.C. (million/ml)
O (n=9)	39.3±7.2	9.5±3.8
A (n=7)	10.5±4.5	5.6±2.6
OA (n=7)	9.1±6.8	33.5±10.3
AZ (n=4)	–	–
NZ (n=13)	58.75±7.8	65.5±25.4
Controls (n=40)	67.43±10.3	114.5±44.8

[O –Oligozoospermia, A – Asthenozoospermia, OA – Oligoasthenozoospermia, AZ – Azoospermia, NZ – normozoospermia]

Reactive Oxygen Species (ROS) Quantification:

ROS levels were measured by luminol-induced chemiluminescence in semen of cytogenetically normal, non-azoospermic infertile men (n=36) and controls (n=40). The ROS was estimated in duplicate each time, and the mean of 3 readings done over a 3-week interval was reported. The entire ROS estimation procedure was done by the same individual to minimize inter-user variability.

In cases with cytogenetic abnormality and Yq microdeletion, the sperm count was either zero

or very low (< 1 million/ml). Since the chemiluminescent method for ROS estimation is not sensitive for semen samples with such a low count [18], it was not possible to estimate ROS levels in these samples.

The neat semen ROS levels were significantly higher (p<0.05) in all categories of infertile men as compared to controls. In infertile men, neat semen ROS values ranged from 14.76 ± 1.86 RLU/sec/million to 67.6 ± 5.78 RLU/sec/million whereas in controls, it was 1.246 ± 1.67 RLU/sec/million to 24.34 ± 3.48 RLU/sec/million.

The mean neat semen ROS in infertile men was 31.7418 ± 4.86 RLU /sec/million compared to 12.45 ± 2.68 in controls (cut off value of ROS is d"22 RLU/sec/million sperm). In 74.48 % of infertile men, neat semen ROS levels were higher than the critical value (d"22 RLU/sec/million).

Fig. 1: ROS values in cases (n=36); cut off value- 22 RLU/sec/million.

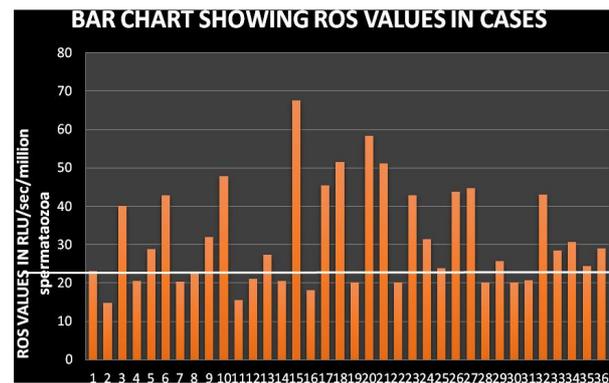


Fig. 2: ROS values in controls (n=40); cut off value-22 RLU/sec/million.

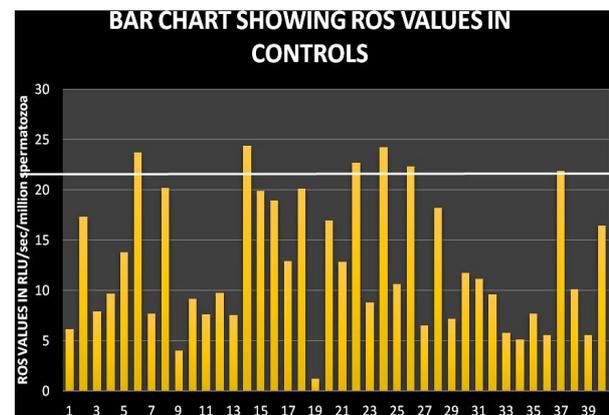


Table 3: ROS levels in cases and controls (mean ±S.D.) (p<0.05).

	Cases	Controls
ROS values (RLU/sec/million)	31.7418 ± 4.86*	12.45 ± 2.68*

Table 4: Comparison of study parameters in cases and controls.

Study parameters	Case (n=36)				Control (n=40)				't'	'p' value
	Mean	SD	Range		Mean	SD	Range			
			Min	Max			Min	Max		
Motility A	15.43	10.6	0	30	29.87	14.4	10	50	5.1	0.01*
Motility B	14.25	12.5	0	30	24.13	11.9	10	55	3.6	0.01*
Motility C	27.75	11.6	0	40	19.88	11.1	10	40	0.76	0.98
Motility D	43.57	16.8	5	95	26.5	13.3	5	55	0.58	0.99
HEAD	19.43	8.7	7	42	15.72	6.3	3	29	2.1	0.037*
MIDPIECE	10.97	5.8	3	23	8.45	4.5	1	23	2.1	0.037*
TAIL	8.97	7.6	0	27	6.1	4.9	0	24	1.9	0.051*
CYTOPLASMIC DROPLET	2.72	3.4	0	17	1.33	1	0	4	2.5	0.016*
SPERM DEFORMITY INDEX	0.42	0.08	0.29	0.56	0.31	0.1	0.14	0.56	5.2	0.00*

(*-significant at p<0.05)

Evaluation of morphological parameters: The slides of both cases and controls were evaluated for morphological defects by WHO, 1999 [9] criteria. The morphological defects are classified as head, mid-piece, tail deformities, cytoplasmic droplets and the score is given as SPERM DEFORMITY INDEX (SDI) and TERATOZOOSPERMIC INDEX (TZI).

Head defects in the cases range from 7 to 42 with a mean of 18.75±1.26 with the most common head defect being the amorphous type (irregular heads). Mid-piece defects range from 3 to 23 with a mean of 10.68±1.74 with the most common defect being thick mid-piece. Tail defects range from 0 to 27 with the mean of 8.73 ± 0.87, the most common tail defect being coiled tails. The cytoplasmic droplets range from 0 to 17 with a mean of 2.70 ± 0.45.

Table 5: Correlation between ROS levels with other morphological parameters.

MORPHOLOGICAL PARAMETERS	'r' VALUE	'p' VALUE
HEAD	0.062	0.723
MIDPIECE	0.439	0.007*
TAIL	0.165	0.337
CYTOPLASMIC DROPLET	0.431	0.009*
SDI	0.72	0.00*

(number of head defects, mid-piece defects, tail defects, cytoplasmic droplets, SDI) in the cases (n=36); 'p' value <0.05 is considered significant (*).

There is a significant difference in all the morphological parameters like head ('t'=2; p<0.05), mid-piece ('t'=2.1; p<0.05), tail ('t'=1.9; p<0.05), cytoplasmic droplets ('t'=2.5; p<0.05) between cases and controls. Sperm deformity index showed highly significant difference ('t'=5.2; p<0.05) between cases and controls.

There is a significant positive correlation between ROS levels and cytoplasmic droplets (r=0.431; p=0.009), mid-piece defects (r=0.439; p=0.07) and SDI (r=0.72; p=0.00) in the cases whereas a significant positive correlation between ROS levels and tail (r=0.326; p=0.04) and SDI (r=0.495; p=0.001) in the controls. Thereby SDI has a positive correlation with the ROS levels in both cases and controls.

Table 6: Correlation between ROS levels with other morphological parameters.

MORPHOLOGICAL PARAMETERS	'r' VALUE	'p' VALUE
HEAD	0.24	0.136
MIDPIECE	0.234	0.147
TAIL	0.326	0.04*
CYTOPLASMIC DROPLET	0.298	0.06
SDI	0.495	0.001*

(number of head defects, mid-piece defects, tail defects, cytoplasmic droplets, SDI) in the controls (n=40); 'p' value <0.05 is considered significant (*).

Multiple regression analysis was carried out by taking ROS levels as dependent variable and other morphological parameters such as head defects, mid-piece defects, tail defects and Sperm deformity index (SDI) as independent variables in both cases and controls.

The regression equation in the cases (n=36) was found to be significant (F=12.51; p<0.001). The regression analysis showed that about 63% of the variation occurring in the ROS levels can be explained by the head, mid-piece, tail defects and cytoplasmic droplets. All these independent variables are directly related to ROS levels. The remaining 37% might be due to some other variables not measured in our study. The regression equation in the controls (n=40) was found to be insignificant (F=3.169; p>0.001).

A simple scatter diagram was drawn between ROS levels and each of the morphological parameters in cases and controls. Each scatter diagram shows linear relationship between ROS levels and morphological parameters. In each of the scatter diagram there seems to be a weak positive correlation (as evidenced by R^2 values) between the morphological parameters and the ROS values.

There is a positive correlation between numbers of mid-piece defects ($R^2=0.192$), cytoplasmic droplets ($R^2=0.185$) and the ROS levels in the cases and number of tail defects ($R^2=0.106$) and the ROS levels in the controls. The cytoplasmic droplets and mid-piece defects might serve as indicator of oxidative stress induced damage in cases (i.e., those with elevated ROS levels) but not in controls (i.e., normal or low ROS levels).

The SDI shows a strong positive correlation ($R^2=0.518$) with the ROS levels in the cases and in the controls ($R^2= 0.244$) too. This makes evident that SDI is the best possible indicator for the oxidative stress induced damage both in cases and controls.

Fig. 3: Scatter diagram showing the linear relationship between ROS levels and the sperm deformity index (SDI) in cases (n=36); $R^2=0.5183^*$.

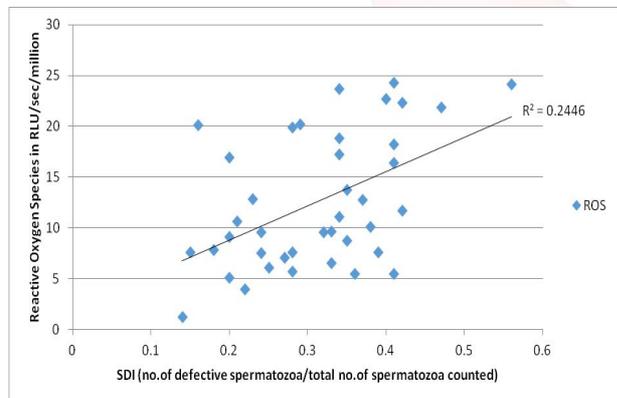


Fig.4: Scatter diagram showing the relationship between ROS levels and the sperm deformity index (SDI) in controls (n=40); $R^2=0.2446$.

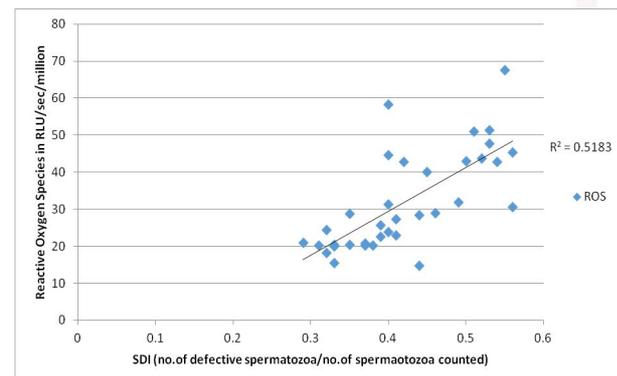


Fig. 5: H/E stained image (60 X) showing cytoplasmic droplets (Scale Bar-50 μ m).

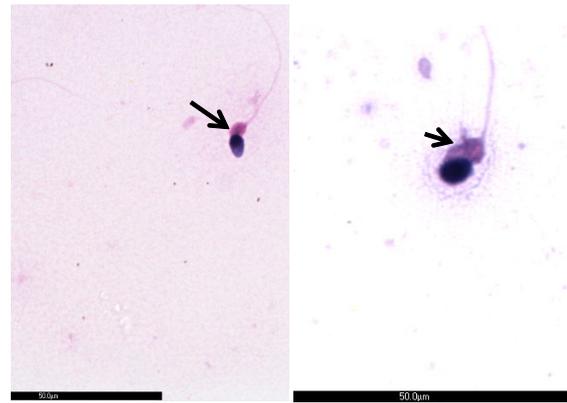
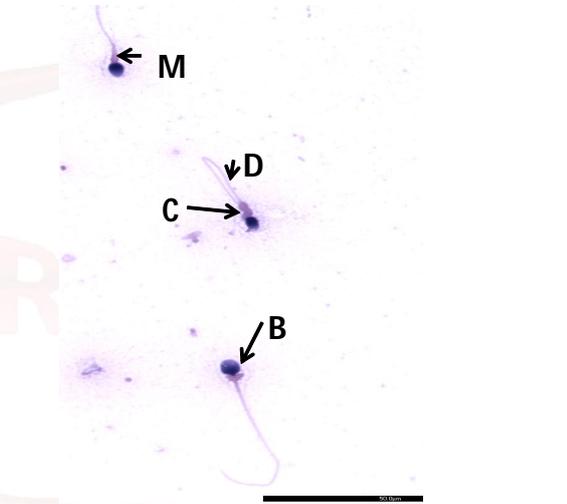


Fig.6: H/E image (60X) Showing B-Pyriiform Head, C-Cytoplasmic Droplet D-Coiled Tail, M-Midpiece Defect (scale bar-50 μ m).



DISCUSSION

Male infertility is considered to be a multifactorial disorder affected by genetic, environmental and hormonal factors. It is a common, complex disorder and recent studies have called infertility a lifestyle disease, due to role of various lifestyle factors which adversely affect male reproductive health. Recent studies have emphasized the role of oxidative stress and sperm DNA damage (denaturation and fragmentation) as a cause of infertility. The reports emphasize an association of excessive ROS generation with DNA strand breaks [19,20] and chromosome deletions in spermatozoa [21]. These changes may not be detected by routine semen analysis, although they can cause reproductive failure. In this study, we evaluated men who presented with idiopathic infertility for ROS levels and for morphological defects in the spermatozoa.

Oxidative stress induced sperm damage has

been suggested to be a significant contributing factor in 30–80% of cases of male infertility [22]. High ROS levels may lead to fertilization failure due to alteration of sperm membrane permeability and fluidity and also cause pronuclear block or slow cleavage and may be one of the factors responsible for low success rate in assisted conception [23].

In this study, we used an indirect luminol-dependent chemiluminescence assay to measure the levels of ROS in neat semen. Luminol or lucigen probes can be used for quantification of redox activities of spermatozoa; they have well established reported ranges for fertile and infertile populations thus bringing clinical relevance to its use [24]. Luminol measures both intracellular and extracellular ROS in the semen. Chemiluminescence may be accurate and reliable but only in samples with sperm concentration >1 million/ml [18]. We had included only those cases with sperm concentration >1 million/ml for this study and the ROS levels had been measured within 30 minutes after ejaculation. The samples were repeated thrice within 2 weeks interval. As ROS levels fluctuate in this study, a mean of 3 readings has been taken.

In this study, we had observed that the mean ROS levels in cases (31.7418 ± 4.86 RLU /sec/million) was significantly ($p < 0.05$) higher than the mean value in the controls (12.45 ± 2.68 RLU/sec/million). 23 out of the 36 cases and 6 out of 40 controls had ROS levels more than the critical value ($d > 22$ RLU/sec/million spermatozoa). One of the previous studies in our laboratory [25] had established interquartile range of ROS levels in fertile controls as $0.014 - 0.11 \times 10,000$ RLU/min/20 million sperm (22 RLU/sec/million spermatozoa) which is similar to the findings by *Fingerova et al.*, [26] $0.12 - 0.55 \times 1000$ RLU/min/20 million sperm. It has also been observed that the % DNA fragmentation index (DFI) was significantly higher (32.88 ± 7.41) in infertile men with elevated ROS levels compared with controls (22.50 ± 2.81) with normal ROS levels [27].

A meta-analysis suggests that ROS levels are negatively correlated with fertilization rates following IVF (estimated overall correlation = 0.374; [95% CI, 0.520, 0.205]) [28]. It is stated that patients with normal semen parameters may

have high levels of ROS that may affect the fertilizing capacity of spermatozoa [29]. We also observed that ROS levels in 13 infertile men had normal ROS levels (i.e., $d > 22$ RLU/sec/million). It is already implied that physiological levels of ROS are required for sperm capacitation, acrosome reaction, and fertilization, therefore low ROS levels could be factor contributing to infertility in these men. This observation implies that antioxidant supplementation should not be administered indiscriminately as an empirical treatment for idiopathic infertility; rather only the cases with supra-physiological ROS levels should be considered for such therapy. In this study, to the best of our knowledge we had excluded the cases with systemic infection, epididymitis and seminal vesiculitis, fever and recent drug intake (<3 weeks), thereby eliminating the overt leukocyte contamination. This indicates that the observed high ROS level in infertile men was due to elevated ROS production by immature/pathological sperm rather than by leukocytes. Increased mitochondrial DNA mutations, dysfunction of mitochondrial oxidative phosphorylation, and electron leakage from the electron transport chain within the sperm mitochondria could be possible sources of higher ROS levels in the infertile men [30, 31]. We also made an attempt to correlate various inter-morphologic defects with ROS levels, to find out the best morphologic indicator of oxidative stress.

The multiple-entry technique used in our study [12] ensures that deformities of different parts of the sperm are accounted for equally. This enables us to evaluate the influence of specific sperm structural deformities, alone or in combinations, on sperm function. It also allows the calculation of the SDI.

In this study, we had found significant positive difference in cytoplasmic droplets ($p = 0.016$), head defects ($p = 0.037$), mid-piece defects ($p = 0.037$) between cases and controls. The tail defects did not show much significant difference ($p = 0.051$) between cases and controls. Sperm deformity index (SDI) had the most significant ($p = 0.00$, $t' = 5.2$) difference between cases and controls. This was in accordance to *Said TM et al.*, [21] who suggested the use of SDI to evaluate infertile group and it has been found to

distinguish the semen sample with impaired fertility and Aziz N et al., [12] who had showed a positive correlation of ROS with SDI. ($r=0.51$, 95% CI=0.3 to 0.7; $P=0.0001$).

Our study was in accordance with Aziz N et al., who had showed a significant positive correlation between sperm ROS production and the proportion of sperm with mid-piece defects ($r=0.45$, 95% CI=0.2 to 0.65; $P=0.0006$), cytoplasmic droplets ($r=0.28$, 95% CI=0.008 to 0.51; $P=0.04$), tail defects ($r=0.47$, 95% CI=0.23 to 0.66; $P=0.0005$) and SDI ($r=0.51$, 95% CI=0.3 to 0.7; $P=0.0001$).

No significant correlation was observed between levels of seminal ROS and the proportions of spermatozoa with head defects. This could be explained by the fact that the spermatozoa is a highly compartmentalized structure and as a consequence of this arrangement, the endonucleases released and activated during apoptosis remain resolutely locked in the mid-piece of the cell and never gain access to the nucleus [32]. Even if an endonuclease did manage to gain access to the sperm nucleus, it would take some time to permeate such a dense structure and induce widespread DNA damage [33]. It can be concluded that unless there is a primary defect in the chromatin remodelling during spermiogenesis, the chance of DNA damage is purely oxidative by the ROS leaked from the mitochondria. The significant correlation between sperm ROS production and the proportions of spermatozoa with cytoplasmic droplet ($r=0.431$; $p=0.009$) and mid-piece defects ($r=0.439$; $p=0.007$) demonstrated in the infertile men is in agreement with the studies [34,35]. These functionally defective, vulnerable, free radical-generating, DNA-damaged, apoptotic cells exhibiting cytoplasmic retention and a high polyunsaturated fatty acid content probably correspond to the 'immature' cells described by Huszar's group [36,37]. These may also be the cells which escape apoptotic cascade due dysfunctional mitochondria. Mitochondria are both source and target of free radicals and one of the first sites of oxidative damage.

Gil-Guzman E et al., [35] proposed that the correlation of mitochondrial membrane potential (MMP) with sperm morphology might provide interesting information as morphologically

abnormal spermatozoa with mid-piece defects have been linked with excessive production of ROS. Xia Wang et al., [38] obtained a significant positive correlation was seen between MMP and sperm concentration ($r=0.62$, $P<.001$). The MMP was inversely correlated with ROS levels ($r=0.45$, $P<.05$). Therefore the increased number of mid-piece defects in our study might be due to the dysfunctional mitochondria which might then lead to excessive ROS production.

A central tenet of the hypothesis proposed by Aitken RJ and De lullis, [39] is that, in a majority of cases, the ROS that attack the DNA come from the spermatozoa themselves and specifically, their mitochondria. These poorly remodelled cells bear many of the hallmarks of cellular immaturity, particularly the retention of excess residual cytoplasm resulting in elevated cellular levels of several biochemical markers for the cytoplasmic space including creatine kinase, glucose-6-phosphate dehydrogenase, superoxide dismutase and lactic acid dehydrogenase [34,36,40]. In our study, the regression studies had shown that 63% of the variation in the ROS levels can be attributed to the morphological defects in the infertile men and 27% of the variation in the ROS levels can be attributed to the morphological defects in controls.

Sperm deformity index (SDI) scores in our study showed significant positive correlation with the sperm ROS production both in cases ($r=0.72$; $p=0.00$) and in controls ($r=0.495$; $p=0.001$) suggesting it as a single most important predictor for ROS-mediated sperm damage. According to Said et al., (21) samples with higher SDI scores had higher increase in DNA damaged sperm compared to those with lower SDI scores ($p=0.04$). In another study by Aziz et al., [41] the non-apoptotic sperm subpopulation had morphologically superior quality sperm compared with apoptotic sperm as reflected by significantly lower SDI scores as compared to apoptotic sperm fraction. In our study also SDI showed a positive linear relationship with ROS levels ($R^2=0.518$) in the cases.

CONCLUSION

Through this study, we envisage that the genetic analysis of infertile men supplemented with assessment of neat ROS levels and morphologi-

cal defects may provide us more direct evidence of the risk and help to appropriately counsel patients regarding the prognosis of assisted conception. The positive correlations with the proportion of sperm with morphological defects, SDI scores with sperm ROS production show that morphological evaluation (including SDI) serves as a useful tool in identifying infertile men with high seminal ROS levels in infertility clinics where dedicated, expensive laboratory facilities for applying labour intensive techniques are not available.

Oxidative stress can be minimised to a certain extent by lifestyle modifications and usage of systemic antioxidants for the management of selective cases of male infertility (with supra-physiological ROS levels) as well as in vitro supplements during various sperm preparation techniques.

Conflicts of Interests: None

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