

## SHORT COMMUNICATION

**Clinical significance of reactive oxygen species in semen of infertile Indian men**S. Venkatesh<sup>1</sup>, A. M. Riyaz<sup>2</sup>, M. B. Shamsi<sup>1</sup>, R. Kumar<sup>3</sup>, N. P. Gupta<sup>3</sup>, S. Mittal<sup>4</sup>, N. Malhotra<sup>4</sup>, R. K. Sharma<sup>5</sup>, A. Agarwal<sup>5</sup> & R. Dada<sup>1</sup>

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**Summary**

Reactive oxygen species (ROS) levels in semen are believed to play both physiological and pathological roles in male fertility. The study was aimed to find the clinical significance of ROS levels in infertile Indian men. This pilot study included 33 idiopathic infertile men and 18 proven fertile controls. ROS levels in the washed sperm were measured using chemiluminescence assay and expressed as 10<sup>6</sup> cpm per 20 million spermatozoa. Sperm count, percent sperm motility, and percent normal sperm morphology were found to be significantly ( $P < 0.0001$ ) reduced in infertile men compared with the controls. Median (minimum, maximum range) ROS levels of the infertile group [24.90 (6.89, 44.71)] were found to be significantly ( $P < 0.0001$ ) elevated compared with the fertile controls [0.167(0.15, 2.78)]. No significant correlation was seen between ROS levels and semen parameters. Elevated ROS levels in the idiopathic Indian infertile men may be one of the underlying reasons for impaired fertility. Therefore measurement of seminal ROS levels may be used in Indian infertile men for better understanding of the aetiology and selection of antioxidant regimen in the treatment of male infertility. However, large studies may be urgently warranted to find out the role of antioxidants in ROS elevated Indian infertile men through randomised, controlled clinical study.

**Introduction**

One of the major concerns among the male reproductive health is the inability to achieve parenthood even after regular unprotected intercourse. Infertility affects 15% of the married couples and in about 50% of cases male factor is the predominant causative factor (Seshagiri, 2001; Sharlip *et al.*, 2002), where both qualitative and quantitative defects are seen in sperm production. Though standard semen analysis is routinely used in the evaluation of male infertility, the exact mechanism behind the poor semen parameters is rarely understood. Varicocele (Dada *et al.*, 2007), cryptorchidism (Dada *et al.*, 2002), hypogonadism (Isidori *et al.*, 2008) and infection (Ochsendorf,

2008) are found to be the leading causes of infertility but approximately 30–40% is found to be idiopathic (Hellani *et al.*, 2006). Genetic (nuclear and mitochondrial) alteration has also been focused as an important aetiology in male infertility (Kumar *et al.*, 2007; Dada *et al.*, 2008). However, recently the pathological role of elevated reactive oxygen species (ROS) levels in the semen has been focus of several studies in the evaluation of male infertility (Aikten *et al.*, 1993; Desai *et al.*, 2008). ROS are highly reactive oxidising agents belonging to the class of free radicals. ROS at low level facilitate hyperactivation, capacitation, acrosome reaction, motility, fertilisation and oocyte adhesion of spermatozoa (Kodama *et al.*, 1996; Rivlin *et al.*, 2004; de Lamirande & O'Flaherty, 2008;

Venkatesh *et al.*, 2009), but higher ROS damages a variety of biomolecules such as lipids, amino acids, carbohydrates, protein and DNA and adversely affect the sperm function (Sikka, 1996). Recent studies have focused the impact of free radicals on male fertility as they are believed to cause sperm dysfunction either by damaging sperm plasma membrane or DNA. Studies by Agarwal *et al.*, (2006, 2008) reported that ROS levels are elevated in semen of infertile men. The oxidative phosphorylation system of mitochondria is suspected to be both the production and target site of ROS, as it is closely associated with the inner mitochondrial membrane (Beckman & Ames, 1998). Moreover increased free radicals and accumulation of mitochondrial DNA (mtDNA) mutation have also been associated with increase in age (Linnane *et al.*, 1989). Though sperm DNA are packed compactly with protamine by modifying the histones during spermatogenesis, defective protamination may also increase the chance for oxidative DNA damage of spermatozoa (Oliva, 2006). So it is vital to detect the seminal oxidative stress (OS) at the earliest in the male reproductive evaluation to prevent/treat OS-associated male infertility. As spermatozoa with normal morphology and motility are most important predictors of fertility potential in men, OS induced lipid peroxidation also damage sperm membrane affecting its fluidity and motility (Kasperczyk *et al.*, 2008) and can also result in sperm DNA damage. As free radicals are produced as by-product of oxidative phosphorylation pathway at mitochondria, mtDNA are the first site of ROS mediated DNA damage. An earlier study in our lab (Kumar *et al.*, 2007) reported increased frequency of mitochondrial nucleotide substitutions in men with idiopathic infertility. There are no reports that document the availability of ROS measurement as a routine diagnostic method in the infertility clinic in India. Therefore in this study we made an attempt to establish ROS measurement as an important tool in the diagnosis of seminal OS in infertile Indian men. The objective of our study was to (i) examine the ROS level in the spermatozoa of idiopathic infertile men and fertile controls and (ii) to correlate the ROS levels with the semen parameters.

## Methods and materials

Following approval from the institutional ethical committee, AIIMS, New Delhi, India participants were enrolled in the study.

### Study population

The study included 33 idiopathic infertile men and 18 proven fertile controls. Each subject completed a questionnaire which comprised medical, surgical, reproductive

and occupational history and life style factors. Cases with any history of prolonged illness, drug intake, smoking or alcohol consumption were excluded. In addition, these men were cytogenetically normal and had no Yq microdeletions or sperm mtDNA mutations. Physical examination was performed to exclude cases with known factors such as varicocele, cryptorchidism, endocrine disorders etc. Azoospermic, severe oligozoospermic ( $<2 \times 10^6$  per ml), leucocytospermic and highly viscous samples were excluded from the study. Men with history of smoking, alcohol consumption were also excluded from the study as these factors can lead to increased ROS production and mimic tissue inflammation.

### Sample collection and semen analysis

Semen samples were collected in a sterile plastic container after sexual abstinence of 3–5 days and delivered to the laboratory within 30 min. Each sample was incubated at room temperature and after liquefaction the standard semen analysis was performed according to guidelines of the WHO (1999). Percent sperm progressive motility was calculated by recording the sum of percent of grade 'a' and grade 'b' motility. Sperm morphology was recorded by scoring the percentage normal and abnormal forms.

### Estimation of ROS by chemiluminescence assay

Levels of ROS were measured in fresh washed sperm suspensions using a chemiluminescence assay (Athayde *et al.*, 2007). With this protocol, liquefied semen was centrifuged at 300 g for 7 min, and after washing the pellet with phosphate-buffered saline (PBS) it was resuspended in the same washing media at a concentration of  $20 \times 10^6$  sperm per ml. Four hundred microlitre aliquots of the resulting cell suspensions were used to assess basal ROS levels. Ten microlitre of luminol (5-amino-2,3,-dihydro-1,4-phthalazinedione; Sigma), prepared as 5 mM stock in dimethyl sulphoxide, was added to the mixture and served as a probe. A negative control was prepared by adding 10  $\mu$ l of 5 mM luminol to 400  $\mu$ l of PBS. Levels of ROS were assessed by measuring the luminol-dependant chemiluminescence with the luminometer (Sirius, Berthold Detection Systems GmbH, Pforzheim, Germany) in the integrated mode for 15 min. The results were expressed as  $\times 10^6$  counted photons per minute (cpm) per  $20 \times 10^6$  sperm.

### Statistical analysis

All the sperm parameters, age and ROS values between the groups were expressed as median (minimum range, maximum range). The significant difference of these

parameters between the infertile and control group was calculated using two-sample Wilcoxon rank-sum (Mann–Whitney) test and by Student's *t*-test. The correlation between sperm parameters and ROS level was found using Pearson correlation coefficient method. The statistical analyses were performed using STATA 9.0 version (StataCorp LP, TX, USA) software. A *P*-value <0.0001 was considered as significant unless otherwise stated.

## Results

Out of 51 semen samples, this preliminary study included 33 primary infertile cases and 18 proven fertile controls. All the semen parameters including sperm count, percent progressive motility and percent normal sperm morphology of infertile men were significantly ( $P < 0.0001$ ) reduced when compared with the fertile controls (Table 1). However, ROS levels ( $10^6$  cpm) in the semen of the infertile men expressed as median (minimum, maximum range) 24.90 (6.89, 44.71) were significantly ( $P < 0.0001$ ) elevated compared with the control group 0.167(0.15, 2.78) (Table 1). No significant difference was observed in the age between the infertile ( $30.39 \pm 4.40$ ) and control ( $31 \pm 3.32$ ) groups. No correlation was found between the ROS levels and any of the semen parameters of infertile men (Table 2). Age and duration of infertility did not show any correlation with the ROS levels in infertile men.

## Discussion

Though semen analysis is still considered as the fundamental diagnostic step in the evaluation of male infertility, it fails to detect the fertilisation capacity of the sperm. Studies have reported normal semen parameters in infertile men and also men with low sperm quality are able to procreate (Dandekar *et al.*, 2002; Thangaraj *et al.*, 2003). Increased incidences of idiopathic cases of male infertility have focused the attention of reproductive specialist towards other factors responsible for infertility. As Macleod (1943) revealed the role of free radicals in regulating sperm function, the toxic effect of excess free radicals on the sperm cells has gained much importance. Though it is well known that generation of ROS in human spermatozoa is necessary for the activation of tyrosine phosphorylation associated with capacitation for normal fertilisation (Rivlin *et al.*, 2004) its elevated production in some known and unknown pathological conditions have severe detrimental effects on sperm function. As our results showed a significant difference in all the semen parameters between the infertile men and the fertile controls the exact cause for this difference is not known. Hence in these idiopathic cases, we attempted to see the difference in the levels of ROS in the semen ejaculate between the fertile and infertile group. Excess ROS levels in the ejaculate not only damage the sperm plasma membrane rich in polyunsaturated fatty acids but is also believed to affect the sperm DNA (Kumar

**Table 1** Comparison of sperm parameters, ROS level and age of infertile and fertile controls

Subjects	Sperm count $\times 10^6$ per ml	% Progressive motility	% Normal morphology	ROS $\times 10^6$ cpm per 20 million sperm	Age (years)	DI (year)
Infertile ( $N = 33$ )	15 (2, 62.6)	32 (0, 60)	25 (14,70)	24.90 (6.89, 44.71)	$30.39 \pm 4.40$	$5.60 \pm 3.77$
Controls ( $N = 18$ )	66 (22, 222)	60 (46, 90)	70 (53, 90)	0.167 (0.15, 2.78)	$31.00 \pm 3.32$	–
<i>P</i> -value	<0.0001	<0.0001	<0.0001	<0.0001	NS	–

Values are expressed as median (minimum, maximum range) by Mann–Whitney test,  $P < 0.0001$  is considered as significant,

NS, non-significant by student's *t*-test.

ROS, reactive oxygen species; DI, duration of infertility.

**Table 2** Comparison of correlation coefficient between sperm parameters, ROS and DI of infertile men

Parameters	Sperm count	Percent progressive motility	Percent normal morphology	ROS	Age (year)	DI (year)
Sperm count	1.000	–0.2254	0.2093	0.1624	–0.1787	0.0134
Percent progressive motility	0.2254	1.000	0.2759	0.0574	0.2157	–0.0897
Percent normal morphology	0.2093	0.2759	1.000	0.3193	–0.0066	–0.2279
ROS	0.1624	0.0574	0.3193	1.000	0.0211	0.2554
Age (year)	–0.1787	0.2157	–0.0066	0.0211	1.000	–0.0880
DI (year)	0.0134	–0.0897	–0.2279	0.2554	–0.0880	1.000

No significant correlation between any of the parameters; ROS, reactive oxygen species; DI, duration of infertility.

*et al.*, 2007). In addition, ROS may also affect the sperm axoneme by affecting microtubular assembly, inhibit mitochondrial function and affect the synthesis of DNA, RNA and proteins (de Lamirande & Gagnon, 1992). Moreover, impaired sperm motility may also result from decreased axonemal protein phosphorylation and sperm immobilisation and/or by inhibiting the activity of enzyme glucose-6-phosphate dehydrogenase (Maneesh & Jayalekshmi, 2006). Our study found no correlation between any of the semen parameters and ROS levels in infertile men. Similar findings were also reported (Whittington *et al.*, 1999; Pasqualotto *et al.*, 2000). Moreover, total antioxidant capacity (TAC) of the seminal plasma has also been shown to be significantly lower in infertile cases compared with fertile cases (Mahfouz *et al.*, 2008). Even a new measure of ROS-TAC score evaluated by Sharma *et al.*, (1999) has been found to be significantly lower in infertile men compared with the fertile controls. Another important factor that influences the ROS production is the age of the patients. Accumulation of free radicals, damaging to mtDNA and increased production of free radicals by defective mtDNA is a well known theory in the ageing of men and mitochondrial disorders (Taylor & Turnbull, 2005). In our present study, we could not establish any correlation between age and seminal ROS levels. Increased industrialisation, pollution, work stress and other associated factors at the earliest reproductive age could be considered in such cases. However, though several confounding factors can increase ROS production, such as leucocytospermia, infection, smoking, alcohol consumption, exposure to occupational hazard and chemicals (Tremellen, 2008) all such factors were excluded and therefore these factors are not affected. Studies also reported that exposure to artificially produced ROS causes modification of bases, induces deletions, frame shift mutation, DNA cross links and chromosomal defects in the sperm (Kemal Duru *et al.*, 2000).

In a study conducted in an Australian population, the ROS value has been shown to be 4-fold higher in infertile men than in the control population, measured using nitro blue tetrazolium assay. (Tunc *et al.*, 2009) However, in our study ROS median values were approximately 150 times higher in infertile men compared with controls. Though ROS levels have significant impact on male fertility, the levels may vary in different populations. A recent study from Japan reported significantly higher ROS levels in non-pregnant ( $n = 48$ ) group compared with pregnant group ( $n = 41$ ). The study was conducted during the first visit of the patient and they were later followed up to 24 months for pregnancy. Hence the ROS detection was found important in prognostic value for idiopathic male infertility in Japan population. (Yumura *et al.*, 2009). Moreover, a large number of studies in US population have already shown significantly higher ROS in the semen

of infertile cases compared with fertile controls (Pasqualotto *et al.*, 2000, 2008; Agarwal *et al.*, 2006). Another recent study from Brazil hypothesised that increased ROS levels in whole ejaculate positively correlated with age of fertile men, suggesting that delayed fatherhood may reduce chances of pregnancy (Cocuzza *et al.*, 2008). A similar study in Iranian population also showed significantly higher mean ROS levels in infertile patients when compared with controls (Moein *et al.*, 2007). A study from Canada reported increased ROS levels in the semen of 40% infertile men (Iwasaki & Gagnon, 1992). However, in our population all infertile men included in the study were found to have elevated ROS levels in the semen. Therefore, it may be necessary to conduct such type of study in various populations with a large number of samples, which may help in categorising and selecting antioxidant regimen.

Moreover, ROS has also been implicated to cause mutation or polymorphism in both nuclear and mtDNA of spermatozoa (Spiropoulos *et al.*, 2002; Sharma *et al.*, 2004). Recently the OS in the semen has also been assessed by estimating a stable product 8-isoprostane which is formed in cell membranes due to free radical damage to arachidonic acid. The level of the 8-isoprostane has been found to be significantly higher in all infertile patients compared with the controls (Khosrowbeygi & Zarghami, 2007). Though MDA, 8-OHdG and 8-isoprostane are used for indirect OS estimation, measurement of ROS level using chemiluminescence in the semen has gained much importance because it is easy, rapid and reliable. As our direct estimation of ROS in the infertile men showed a significantly higher value compared with the controls, the origin of ROS is not clear, but assumed to be from defective oxidative phosphorylation, low level of leucocytes and immature germ cells in the semen. The elevated ROS levels in the infertile men compared with the fertile group in this study are a clear indicator of OS. Thus early detection of raised ROS level and prompt treatment may not only aid in improvement of sperm function but also may prevent sperm from undergoing irreversible DNA damage. In a study, idiopathic infertile men administered with lycopene (2000  $\mu\text{g}$ ) twice a day for 3 months showed a significant increase in sperm concentration, motility and morphology (Gupta & Kumar, 2002). A study reported that the supplementation of antioxidant rich diet improved semen quality (Eskenazi *et al.*, 2005). Glutathione administration for 2 months on alternate days at a dose of 600 mg (i.m) reported an overall increase in motility, progressive motility, velocity and linearity and also decreased morphologically abnormal sperm in patients with varicocele and those with 'germ-free genital tract inflammation' that were associated with ROS production (Lenzi *et al.*, 1993). Moreover, the

percentage of sperm DNA fragmentation has been found to be markedly reduced after daily administration of 1 g vitamin C and 1 g vitamin E for 2 months (Greco *et al.*, 2005). In the era of ART, the selection of sperm with DNA integrity is mandatory to prevent the transmission of genetic defects to the offspring. Our preliminary study gives a clear picture of OS in idiopathic infertile Indian men. Thus it is also important to study DNA integrity in men with OS if such men opt for ART.

In conclusion, men with idiopathic infertility have elevated ROS levels in the semen and these adversely affect sperm function. These elevated ROS levels may be due to, (i) mainly by defective oxidative phosphorylation at the sperm mitochondria level due to mtDNA mutations, and (ii) low level of leukocytes and immature germ cells in the semen of idiopathic infertile men. In addition, as nucleotide changes are population-specific, it is necessary to define a cut-off value for ROS levels in infertile men of different population. Hence early diagnosis of such OS-associated idiopathic conditions and their impact on male fertility could be useful in the better treatment/selection of antioxidant for improving conception and ART success rate. Further sperm DNA fragmentation studies are warranted in those cases with elevated ROS levels if they are selected for intracytoplasmic injection. Hence, clinical ROS measurement may play an important role in identifying patients with seminal OS and may get benefit from suitable antioxidant therapy. However, selection of suitable dose and type of antioxidants are still to be clinically evaluated, which is urgently needed for better management of excess ROS mediated infertility.

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