Iranian Journal of Reproductive Medicine Vol.7. No.1. pp: 29-34, Winter 2009

Correlation of sperm morphology and oxidative stress in infertile men

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Received: 22 October 2008; accepted: 10 March 2009

Abstract

Background: Excess reactive oxygen species (ROS) in the semen is believed to affect fertility in men. Morphologically abnormal sperms and their relation to seminal oxidative stress in infertile and subfertile men are not clear.

Objective: To correlate various sperm morphological defects with seminal oxidative stress in infertile and subfertile men.

Materials and Methods: The study included 25 primary, 21 secondary infertile men of idiopathic infertility and 15 fertile controls. Standard semen analysis was performed according to WHO (1999) guidelines. Sperm inter-morphological defects were evaluated in 100 sperms per sample by Giemsa staining. ROS in spermatozoa was measured by the chemiluminescence assay.

Results: Significant difference in percent sperm amorphous head was found between secondary infertile group and control men. The study showed a significantly higher percent spermatozoa with residual cytoplasm between primary [11.61 (6.6, 3.9)], secondary [7.49 (0.8, 13)] and fertile controls [2.44 (0.8, 3.7)] similar to sperm count, percent sperm progressive motility, and ROS levels. A non significant but strong positive correlation (r=0.3479, p=0.0884) between percent cytoplasmic retained spermatozoa and ROS levels was observed in the primary infertile group. However, no correlation between other sperm morphological defects and oxidative stress was observed.

Conclusion: Sperm morphology was not found to be associated with oxidative stress in the present study. However, retained cytoplasmic residues in the sperm may be an important source of ROS in both primary and secondary infertile men. These immature spermatozoa are believed to be associated with impaired fertility.

***Key words:** Infertility, Oxidative stress, Spermatozoa, Reactive Oxygen Species.*

Introduction

Declining male reproductive health is a major concern among the population of reproductive age (1).

Sperm counts are falling at an alarming rate of 1% per annum for the last 10 years and concomitant with this is a decline in percent of morphologically normal sperm. Though semen analysis is the first diagnostic step routinely employed in the evaluation of the male infertility, it fails to predict the exact cause behind impaired fertility.

However, sperm count and sperm motility are the first and most important predictors of fertility potential rather than sperm morphology. There are number of common causes of male infertility, which includes gene mutations, aneuploides, varicocele, radiation, chemotherapy, genital tract

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infections, and erectile dysfunction (2, 3) and azoospermia factor (AZF) deletions (4). In half of the male infertile patients, the cause is not clear and hence such cases are diagnosed with idiopathic infertility.

In male factors, sperm morphological defects are rarely evaluated for in vivo and in vitro evaluation of male infertility because of unavailability of universal methods, reliability and predictability. Abnormal sperm morphology has been associated with cytogenetic anomalies, reproductive toxicants, smoking etc (5, 6), but its role in idiopathic infertility is not clear.

Though overall sperm morphological defects could give a basic idea about the fertility status, sperm inter-morphological defects may provide some additional information about the idiopathic cases.

Moreover, idiopathic infertile cases are blindly treated and selected for assisted reproductive techniques without understanding the basic mechanism behind the fertility impairment. 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 (7, 8). Recently oxidative stress (OS) has been considered as one of the major factors believed to be involved in idiopathic male infertility. Low levels of ROS are necessary for normal functions of spermatozoa like capacitation, hyperactivation, motility, acrosome reaction, oocyte fusion and fertilization (9, 10). OS is a condition where the production of ROS overwhelms antioxidant levels (11).

Though various sperm morphological defects are routinely evaluated as a part of semen analysis, its correlation with seminal oxidative stress in different infertile population is not clear. For the past two decades the pathological role of ROS in the semen has been studied but not well established because of various possible sources associated with excess production of ROS including abnormal spermatozoa. Several studies (12, 13) reported sperm morphology as the best predictor, whereas another study reported it as a poor predictor (14) of male infertility.

Though studies have been reported an association between increased ROS production and overall abnormal sperm morphology (15, 16), the role of specific inter-morphological defects in association with oxidative stress is unclear. Moreover, it is also important to understand if there are any morphological defects involved in differentiating infertile and subfertile men. So, the present study was aimed to correlate various sperm morphological defects and seminal oxidative stress in idiopathic infertile and subfertile men.

Materials and methods

Study population

The study included 25 primary infertile (PI) and 21 secondary infertile (SI) patients. Primary infertile patients were those unable to conceive their partner with normal female factor. Secondary infertile cases were those experienced at least one spontaneous abortion with normal female partner. 15 fertile men who have fathered in the past 2 years have been included as controls. Patients with varicocele, hypogonadism, obstructive and non-obstructive azoospermia, cytogenetic abnormalities, history of smoking, alcohol, recent drug intake, prolonged illness and exposure to reproductive toxicants were excluded from the study. The study was approved by the ethical committee of All India Institute of Medical Sciences (AIIMS). Subjects were enrolled in the study after obtaining informed consent.

Semen analysis

All the participants were asked to observe sexual abstinence for 3-5 days before collection of semen. Samples were collected in a sterile plastic container and delivered to the laboratory before 30 minutes had elapsed. Semen was incubated at room temperature and standard semen analysis was performed according to WHO (1999) guidelines (17).

For sperm morphology study, 10µl of liquefied ejaculate was placed on the slide and a smear was made using a cover slip. The smear was dried in air and fixed by 90% ethanol. The slide was dipped in the Giemsa stain for 3 – 5 minutes and washed under running tap water and then dried in air.

Classification of spermatozoa morphological defects

Sperm inter-morphological defects were evaluated in at least 100 spermatozoa per sample and defects expressed as a percentage. Six abnormalities of the head (large head, small head, pyriform head, pointed head, double head and amorphous head), two abnormalities of the mid piece (cytoplasmic droplets and bent neck) and four abnormalities of the tail (coiled tail, bent tail, broken tail and double tail) were evaluated in each group of men.

Measurement of ROS by chemiluminescence assay (18)

Fresh liquefied semen was centrifuged at 300 x *g* for 7 minutes and the pellet was washed with phosphate- buffered saline (PBS- pH 7.4). The washed pellet was resuspended in the same washing media at a concentration of 20 x 10^6 sperm/ml.

Ten microliters of 5M luminol (5-amino-2,3,-dihydro-1,4-phthalazinedione; Sigma), prepared in dimethyl sulfoxide (DMSO), was added to the mixture and served as a probe.

A negative control was prepared by adding 10 µL of 5 mM luminol to 400 µL of PBS. Levels of ROS were assessed by measuring the luminol-dependant chemiluminescence with the luminometer (Sirius, Berthold) in the integrated mode for 15 minutes. The results were expressed as 10^6 counted photons per minute (cpm) / 20 x 10^6 sperm.

Statistical analysis

Sperm parameters, percent inter-morphological defects and ROS values between the groups were expressed as the median (minimum range, maximum range).

The significant difference of sperm parameters between the groups was calculated using a Newmann Kuels test.

The overall significance between all the groups in terms of percentage of inter-morphological defects was calculated by using a Kruskal-wallis test, where the significance between any of the two groups was calculated by a Newmann Kuels test. Correlation between the parameters was found using Spearman's correlation co-efficient method.

The statistical analysis was performed using Stata 9.0 version software. In all the cases p<0.01 was considered as significant unless otherwise stated.

Results

Sperm count, percent progressive sperm motility and ROS levels of all the three groups are shown in the table I. Median sperm count was found to be significantly (p<0.001) lower in PI group [15.9 (3.8, 61.8)] compared to SI [57.4 (18, 89)] and control groups [60.2 (45.3, 91.6)]. Similarly, percent progressive sperm motility was also found to have significantly (p<0.001) lower in PI group when compared to SI and control groups as shown in the table I.

Table I. Seminal parameters of infertile and control groups.

Group	Sperm count (x 10^6/ ml)	Progressive motility (%)	ROS (10^6 cpm)
Primary infertile	15.9 (3.8, 61.8) *	13.5 (3.5, 42) *	20.3 (6.89, 35.9) *
Secondary infertile	57.4 (18, 89) *	55 (20, 90) *	3.4 (0.74. 11.2) *
Control	60.2 (45.3, 91.6)	73.6 (44.6, 78.5)	0.164 (0.15, 1.166)

cpm- counted photons per minute, *p < 0.001 is considered as significant compared to controls.

And also significant difference was found in all the above parameters between PI and SI group. However, median (minimum, maximum) range ROS levels in the semen expressed as 10^6 counted photons per minute/20 million spermatozoa was found to be significantly higher in PI group [20.3 (6.89, 35.9)] compared to SI [3.4 (0.74. 11.2)] and controls [0.164 (0.15,1.166)].

Median ROS levels in the semen of PI and SI group was found to be approximately 124 and 20 times respectively higher than the control groups. Among the sperm inter morphological defects, percent sperm with cytoplasmic droplets (CyD) was found to be significantly higher in PI group followed by SI and controls which may be responsible for corresponding seminal ROS levels in their respective groups. However, no other defects were found to differ significantly except percent amorphous head in SI group which was found to be significantly higher compared to control group (Table II).

Progressive sperm motility showed a strong non significant (p=0.0235) negative correlation with ROS levels in PI group. Among the other parameters percent sperm CyD showed strong positive correlation (r=0.3479, p=0.0884) with ROS levels similar to percent sperm pyriform head (PyH) (r=0.4282, p=0.0327) and sperm broken tail (BrT) (r=0.3699, p=0.0687).

However none of the parameters in SI group showed any correlation with ROS levels.

Table II. Comparison of sperm inter-morphological defects between PI, SI, and control groups.

	PI	SI	Control
Head (%)			
Large head (LH)	2.78 (0, 9.5)	2.17 (0, 5.8)	3.5 (0.6, 4.9)
Small head (SH)	4.26 (0, 11.8)	4.07 (0,11.8)	1.86 (0, 4.9)
Pyriform head (PyH)	2.37 (0, 5.7)	2.25 (0, 4.6)	2.72 (1.1, 4.3)
Pointed head (PnH)	6.59 (0.9,15.7)	6.67(0.9,12.5)	3.64 (1.1,4.6)
Amorphous head (AH)	2.9 (0, 4.5)	3.56 (0, 4.5)^{b}	1.86 (0.3, 3.4)
Double head (DH)	0.74 (0, 4.5)	0.28 (0, 1)	0.37 (0, 1.4)
Round head (RH)	0.92 (0, 4.8)	1.73 (0, 6)	1.46 (0, 6)
Mid piece (%)			
Cytoplasmic droplets (CyD)	11.61(6.6,3.9)^{a,c}	7.49(0.8,13)^{d}	2.44 (0.8, 3.7)
Bent neck (BN)	3.96 (0, 12.5)	3.39 (0, 6.5)	4.82 (0, 9.2)
Tail (%)			
Broken tail (BrT)	3.6 (0, 7.4)	3.57 (0, 6)	1.86 (0, 6)
Bent tail (BnT)	5.52 (0.9, 10.8)	5.24 (2.7, 8)	3.8 (0, 8.2)
Coiled tail (CT)	3.83 (0, 8.8)	3.19 (0, 6.1)	2.29 (0.8, 6)
Double tail (DT)	0.47 (0,1.9)	0.50 (0, 1.8)	0.21 (0, 0.8)

PI- Primary infertile and SI- Secondary infertile group, Values are expressed as median (minimum, maximum) range,

$^{a\&d}$ $p<0.0001$ vs. control, ^{b} $p<0.001$ vs. control, ^{c} $p<0.01$ vs. control by two-sample Wilcoxon rank-sum (Mann-Whitney) test.

Discussion

Oxidative stress in the semen has been found to be elevated in infertile men as reported in many studies (19, 20). Since OS is greatly associated with idiopathic infertile men, the exact source of ROS in the semen is still unknown. Though the sperm count and percent sperm motility are the most accessed sperm parameters during infertility evaluation, sperm morphology is rarely considered. It is not clearly known whether morphological defects are linked to ROS production, whereas as other contaminants in the semen like leukocytes, bacteria and immature germ cells produce high ROS levels (2).

Moreover, single sperm may have variousdeformities that may be necessary to evaluate instead classifying as once. Though several studies have reported the association between abnormal sperm morphology and ROS, it has not been extensively used in the diagnostic evaluation of male infertility. Sperm deformity index (SDI) has been used to evaluate infertile group and it has been found to distinguish the semen sample with impaired fertility (21). Therefore, we evaluated detailed morphological defects to find out if they have any role in ROS production.

Sperm with retained cytoplasmic residues were found to be significantly different between the groups similar to their ROS levels. These results are in support of earlier studies showing that abnormal morphology is highly associated with the production of ROS in idiopathic infertile men (22). Moreover, superoxide anion is believed to be the primary free radical produced by the immature spermatozoa (23). However, activation of the NADPH system mediated by abundant glucose 6-phosphate dehydrogenase in the retained cytoplasmic residue of sperm may be involved in the production of ROS (22). Same study reported that ROS production of NADPH has been greatly increased in immature spermatozoa (22). But the role of mitochondria in ROS production cannot be omitted as genomic mutation or alteration in mitochondria may increase ROS production through a vicious cycle (24).

Moreover, abnormal sperm morphology has been reported to be associated with high sperm DNA fragmentation in infertile men (25-27). In our subfertile group, though they have normal sperm parameters, difference in the percent cytoplasmic residues and ROS levels with the PI and control group could give the better understanding in the impaired fertility mechanism. This impaired fertility may be due to production of ROS by the immature cytoplasm retained spermatozoa. Though the percent amorphous head in SI group was significantly higher compared to control, the mechanism is not clear. The capacity of spermatozoa to produce ROS has also been inversely depends on maturational stage (28, 29).

In the present study, no differences have seen in the inter-morphological defects between the PI and SI groups revealed the absence of their role in the ROS production.

Large numbers of studies with large samples are warranted in future for better understanding in this aspect. Though 30% normal sperm morphology is the criteria for normal fertile men (17), the effect of ROS production by abnormal sperm on normal sperm parameters/DNA integrity is not well studied.

Moreover, increased poor sperm morphology has been reported to reduce successful fertilization rates with increased miscarriages even after successful embryo transfer (30). Spermatozoa exposed to ROS for a short period may have less DNA and membrane damage than exposed for a long duration.

Therefore, subfertile population may have less DNA damage, hence these DNA damage may be reversed by neutralizing free radicals like ROS with suitable antioxidant supplements unlike in PI men, who may have high sperm DNA damage due to prolonged presence of OS in semen. Hence SI cases in our study have been found to contain significantly less percent of cytoplsamic retained sperm and ROS levels which could be used to distinguish them from PI men. Treatment with antioxidants has also been reported significant improvement in sperm count, motility and morphology (31).

Therefore, infertile men identified with OS and increased cytoplasmic retained sperm can yet benefit from AO therapy treatment.

A study also reported a significant reduction in abnormal sperm population after glutathione treatment (32). However at the testicular level the elevated ROS may damage the sperm membrane that would result in the altered morphology, which has to be studied in detail.

In conclusion, various sperm morphological defects were not associated with oxidative stress in both PI and SI group.

However, the retained cytoplasmic residues in the sperm may have significant role in the production of ROS and fertility impairment. However, proper work up of such men for both OS and detailed sperm morphological evaluation rather either alone could be useful in treating SI patients with antioxidants to improve their fertilization rate. Because of highly elevated ROS levels and percent cytoplasmic residues in PI men compared to the other groups, antioxidant treatment must be evaluated in detail in the form of clinical trials for the successful pregnancy.

Also ROS at elevated levels have been reported to cause DNA damage, which is one of the suspected causes for poor ART outcome including embryonic cleavage, fragmentation and post implantation loss. Since intra cytoplasmic sperm injection (ICSI) involves the selection of single sperm for fertilization, sperm morphology assessment plays a vital role in sperm selection. Since ROS has also been reported to affect ART, these cytoplasmic residue sperm can be separated to minimize the OS during gamete preparation for ART.

Since cytoplasmic residues has been suspected to increase ROS production, higher sampled studies are required to find their correlation in impaired male fertility.

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