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Does High Load of Oxidants in Human Semen Contribute to Male Factor Infertility?

Hong Chen,[1,*] Hong-Xin Zhao,[1,2,*] Xue-Feng Huang,[1] Guo-Wu Chen,[1] Zhi-Xing Yang,[3] Wei-Jie Sun,[3] Meng-Hua Tao,[4] Yao Yuan,[2] Jun-Qing Wu,[1,2] Fei Sun,[5] Qi Dai,[6] and Hui-Juan Shi[1,2]

Abstract

Basal generation of reactive oxygen species (ROS) was essential for male reproductive function, whereas high ROS levels may be linked to low quality of sperm and male infertility. We examined the associations between ROS levels in whole ejaculates and sperm quality among 1092 male factor infertility (MFI) patients and 50 donors with normal semen characteristics. ROS levels were significantly positively correlated with abnormal morphology rate, head defect, and sperm deformity index. Further, we investigated whether seminal plasma from MFI patients with high ROS levels affects sperm motility from donors with normal semen characteristics. After cross-culturing fresh human sperm from donors possessing normal semen characteristics with seminal plasma from infertile men, sperm motility was measured at different ROS levels. Seminal plasma from MFI patients significantly reduced motility of sperm and the reduction rate increased with increasing ROS levels in seminal plasma. On the other hand, we found MFI patients with the ROS levels in the lowest 25th percentile had similar ROS levels to donors with normal semen characteristics. Collectively, our observations lead to the hypothesis that oxidative stress plays a critical role in the development of MFI among those with high ROS levels, but not those with low ROS levels. *Antioxid. Redox Signal*. 16, 754–759.

Introduction

PREVIOUS STUDIES INDICATED that basal generation of reactive oxygen species (ROS) was essential for male reproductive function, such as initiation of capacitation (4). Conversely, in recent years, extensive studies have been conducted to examine the relationship between oxidative stress (OS) and pathological conditions that affect human spermatozoa. Several prevalent studies observed that high levels of ROS in semen were negatively correlated with sperm motility and normal morphology (8, 2) by using chemiluminescence methods (5). Furthermore, high ROS levels were identified as an independent biomarker of male factor infertility (MFI), irrespective of whether these patients had normal or abnormal semen parameters (3).

One major concern in these previous studies (8, 2) was that ROS levels were determined in washed sperm suspensions,

Innovation

In the largest study thus far, we confirmed that reactive oxygen species (ROS) levels in whole ejaculates were significantly positively correlated with abnormal morphology rate, head defect, and sperm deformity index. To the best of our knowledge, this is the first study to assess the effects of seminal plasma from male factor infertility (MFI) patients with different ROS levels on sperm from donors with normal semen parameters. We found that motility of sperm significantly reduced after treatment with plasma from MFI patients for 6 h. The reduction rate increased with increasing ROS levels in seminal plasma from MFI patients. We also found that MFI patients with the ROS levels in the lowest 25th percentile had similar ROS levels to donors with normal semen characteristics. Our observations lead to the hypothesis that oxidative stress plays a critical role in the development of MFI among those with high ROS levels, but not those with low ROS levels.

[1]Shanghai Medical College, Fudan University, Shanghai, People's Republic of China.
[2]National Population and Family Planning Key Laboratory of Contraceptive Drugs & Devices, Shanghai Institute of Planned Parenthood Research, Shanghai, People's Republic of China.
[3]Huzhou R&D Center for Nutrition and Health, Shanghai Institutes for Biological Sciences, Zhejiang, People's Republic of China.
[4]Department of Medicine, Mount Sinai School of Medicine, New York, New York.
[5]Laboratory for Reproductive Biology, School of Life Science, University of Science and Technology of China, Shanghai, People's Republic of China.
[6]The Vanderbilt Epidemiology Center, Institute for Medicine and Public Health, Department of Medicine, School of Medicine, Vanderbilt University, Nashville, Tennessee.
*These authors contributed equally to the study as the first author.

whereas seminal plasma removed from the process contains high antioxidant components. Furthermore, ROS production by human sperm could be stimulated by centrifugations, resuspensions, and vexing actions utilized during the preparation (1). Using a modified analytic approach, a recent study was able to compare ROS level in neat semen (whole unprocessed) and in washed spermatozoa and found that the ROS in the former was significantly lower than in the latter (6), while another study found that high ROS levels in whole ejaculates were negatively related to sperm curvilinear velocity, linearity, and sperm motility (7). However, these studies had a small sample size. Therefore, in this large-scale study, we confirmed the associations between the ROS levels in whole ejaculates and the presence of abnormal semen parameters and sperm motility. However, in studies with cross-sectional nature, the temporal sequence for the causal association was not entirely clear. Recent *in vitro* studies found that exposure to hydrogen peroxide significantly reduced motion parameters and damaged DNA in healthy human sperm. Nevertheless, hydrogen peroxide may not truly mimic the *in vivo* situation. In the current study, we evaluated the effects of using seminal plasma obtained from MFI patients as a culturing agent on the motility of sperm donated by subjects who had normal semen characteristics. To the best of our knowledge, this is the first study of this kind. In addition, this is the first study reporting ROS levels in whole semen among East Asian populations by using chemiluminescence method and the first study measuring sperm ROS levels among Chinese men.

ROS Levels Significantly Positively Correlated with Abnormal Morphology Rate, Head Defect, and Sperm Deformity Index

Shown in Table 1 are the comparisons of characteristics between 1092 infertile participants and 50 donors with normal semen characteristics. Infertile participants had a median of 4 years of infertility. Although median age was older, donors with normal semen characteristics had a higher overall sperm quality than infertile participants.

We found that 1092 infertile males had significantly higher ROS levels in whole ejaculates than 50 donors with normal semen characteristics ($p < 0.05$; Fig. 1). Further, we found that among 1092 infertile males ROS levels were significantly positively correlated with semen parameters, including abnormal morphology rate, head defect, and sperm deformity index (SDI; see Table 2), but not with motility parameters.

The 95th percentile value for ROS levels among 50 donors with normal semen characteristics was used as the critical value (*i.e.*, 40 RLU/s). The ROS levels for 1092 infertile males were classified into OS-positive (greater than or equal to critical value) and OS-negative (less than critical value) groups. We found the OS-positive group comprised 49.4% of subjects. We compared semen parameters between OS-negative and OS-positive groups (See Table 3). We found that the rates for abnormal morphology, head defect, and SDI were higher among OS-positive group than those among OS-negative group.

MFI Patients with the ROS Levels in the Lowest 25th Percentile Had Similar ROS Levels to Donors with Normal Semen Characteristics

On the other hand, we found that the ROS levels for donors with normal semen characteristics (median 22 RLU/s) were similar to MFI patients with ROS levels in the lowest 25th percentile (*i.e.*, ROS level in Group A; median = 18 RLU/s; data not shown).

Table 1. Baseline Characteristics of 1092 Infertile Participants and 50 Donors

	Infertile males	*Donors with normal semen characteristics*
Number	1092	50
Age, year	30 (28, 34)	35 (31, 37)
Years of infertility, year	4 (3, 6)	N/A
Volume of semen (ml)	2.5 (1.8, 3.5)	3 (2.5, 3.5)
pH	7.2 (7.2, 7.2)	7.2 (7.2, 7.2)
Sperm concentration (10^6/ml)	39.2 (20.4, 65.2)	44.1 (29.2, 82.0)
Motility (%)	51.1 (27.7, 70.6)	77 (72, 85)
Live/dead viability (%)	70.2 (52.0, 83.3)	86.5 (81.0, 92.5)
Abnormal morphology rate (%)	88.3 (71.1, 93.7)	58.1 (52.2, 60.4)
Head defects (%)	80.6 (55.5, 91.0)	52.0 (50.7, 56.5)
Neck/midpiece defects (%)	9.9 (5.9, 14.8)	6.7 (4.1, 9.3)
Tail defects (%)	2.3 (1.0, 5.0)	1.4 (0.7, 3.3)
TZI	1.11 (1.04, 1.16)	1.07 (1.03, 1.12)
SDI	0.93 (0.76, 1.04)	0.63 (0.55, 0.71)
ROS level (RLU/s)	40 (24, 71)	22 (18, 31)

Medians (25th percentile, 75th percentile) were presented except for number.

N/A, data are not available; ROS, reactive oxygen species; TZI, teratozoospermia index; SDI, sperm deformity index; RLU/s, relative light units per second.

Seminal Plasma from MFI Patients Significantly Reduced Motility of Sperm from Donors and the Reduction Rate Increased with Increasing ROS Levels in Seminal Plasma

To evaluate the direct toxic effect of seminal plasma from MFI patients on sperm from donors with normal semen

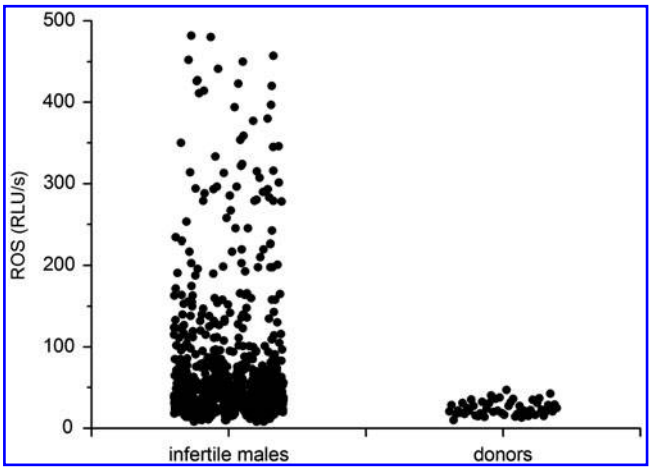

FIG. 1. The ROS levels of 1092 MFI patients and 50 donors. About 1092 infertile males had significantly higher ROS levels in whole ejaculates than 50 donors with normal semen characteristics using the Wicoxon signed rank test ($p < 0.05$). ROS, reactive oxygen species; MFI, male factor infertility.

Table 2. The Associations Between Semen Parameters and Reactive Oxygen Species Levels

ROS level	*Age*	*Volume*	*Liquefied time*	*pH*	*Motility*	*A + B*
Spearman correlation	−0.05350	0.08397	−0.02120	0.01996	−0.00150	−0.00910
p-value	0.3654	0.1415	0.7105	0.7272	0.9637	0.7696
	VCL	VSL	VAP	ALH	LIN	WOB
Spearman correlation	0.04606	0.06001	0.05143	0.03893	0.07699	0.05587
p-value	0.4205	0.2938	0.3684	0.4961	0.1778	0.3284
	STR	BCF	MAD	Viability	Abnormal morphology rate	Head defect
Spearman correlation	0.06711	−0.01430	−0.03030	−0.03040	0.29258	0.36680
p-value	0.2403	0.8030	0.5962	0.3330	0.0098	0.0010
	Body/midpiece defect	Tail defect	TZI	SDI		
Spearman correlation	−0.10300	−0.01180	0.00393	0.26057		
p-value	0.3759	0.9190	0.9731	0.0230		

A + B, motility of class A and class B sperm; VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; ALH, amplitude of lateral head displacement; LIN, linearity; WOB, wobble; STR, straightness; BCF, beat cross frequency; MAD, mean angle of deviation.

characteristics, we performed modified human sperm motility assay (HSMA; see Table 4). Sperm from donors were cultured with HTF (control) or the seminal plasma samples from MFI patients (Group A with the lowest ROS level, Group B with the medium level, and Group C with the highest level). Motility was assessed and sperm motility index (SMI) was calculated. We found that the reduction rates of motility were more pronounced among those with higher ROS levels (*i.e.*, SMI in Group A > SMI in Group B > SMI in Group C; Table 4), although SMI in all groups decreased and the reduction increased with time, and SMI became < 0.75 for all groups after 12 h. The result of repeated measurement analysis of variance showed that the interaction between time and group was statistically significant ($p < 0.05$), indicating that the ROS level plays a role affecting SMI (Table 5).

Conclusions and Open Questions

In the largest study thus far, we confirmed that ROS levels in whole ejaculates were significantly positively correlated with abnormal morphology rate, head defect, and SDI. To the best of our knowledge, this is the first study to assess the effects of seminal plasma from MFI patients with different ROS levels on sperm from donors with normal semen parameters. We found that motility of sperm significantly reduced after treatment with plasma from MFI patients for 6 h. The reduction rate increased with increasing ROS levels in seminal plasma from MFI patients. We also found that MFI patients with the ROS levels in the lowest 25th percentile had similar ROS levels to donors with normal semen characteristics. Therefore, our study indicates that OS plays an important role in the pathogenesis of MFI only among those with high ROS levels.

In most previous studies, washed sperm suspensions were used to evaluate the association between levels of ROS and sperm motility and normal morphology (8, 2). However, washed sperm may not reflect *in vivo* OS status because seminal plasma was considered to be protective for spermatozoa because of its high antioxidant content. On the other hand, some reported that seminal plasma also had negative impacts on sperm structure and function (9). Other studies found that combination of ROS level in semen and total antioxidant capacity (TAC) in seminal plasma was much better than ROS or TAC alone in differentiating between fertile and infertile men. In addition, centrifugation during sperm washing produced additional ROS, especially when the sperm quality was abnormal or when the semen was contaminated with leukocytes (1). A modified method was developed to measure ROS levels in whole (unwashed) ejaculates, without separating the sperm and seminal plasma. ROS in whole ejaculates was strongly

Table 3. Sperm Parameters in Oxidative Stress (OS)-Negative and OS-Positive Groups

Sperm parameters	*OS negative* (n = *553*)	*OS positive* (n = *539*)	p-*value*
Motility (%)	51.9 (28.5, 71.5)	49.7 (26.6, 69.6)	0.3068
A + B (%)	34.3 (17.4, 50.6)	33.8 (15.0, 49.9)	0.2086
Concentration	40.4 (21.2, 65.2)	38.0 (20.0, 65.4)	0.5854
Live/dead viability (%)	71.3 (58.0, 86.0)	69.6 (51.4, 83.0)	0.1031
Abnormal morphology rate (%)	82.0 (56.7, 91.0)	91.2 (76.6, 96.6)	0.0452
Head defect (%)	58.6 (42.3, 82.7)	88.6 (70.7, 91.7)	0.0009
Body/midpiece defect (%)	11.3 (6.5, 15.0)	9.6 (5.3, 14.0)	0.1869
Tail defect (%)	2.8 (1.0, 5.5)	2.0 (1.0, 4.9)	0.4943
TZI	1.1 (1.0, 1.2)	1.1 (1.1, 1.2)	0.7541
SDI	0.84 (0.66, 1.03)	0.99 (0.87, 1.05)	0.0535

Medians (25th percentile, 75th percentile) were presented.
A + B, motility of class A and class B sperm; TZI, teratozoospermia index; SDI, sperm deformity index.

Table 4. Sperm Motility Index at Different Time Points During Human Sperm Motility Assay

	SMI values at different time points (0–24 h)				
Group	*0*	*3*	*6*	*12*	*24*
A (low ROS)					
Donor 1	1.00	0.86	0.82	**0.61**	**0.51**
Donor 2	1.00	0.81	0.77	**0.55**	**0.53**
Donor 3	1.00	0.94	0.81	**0.60**	**0.29**
Donor 4	1.00	0.79	**0.67**	**0.36**	**0.31**
Donor 5	1.00	0.87	**0.68**	**0.54**	**0.33**
Mean	1.00	0.85	0.75	**0.54**	**0.40**
B (medium ROS)					
Donor 1	1.00	0.80	0.75	**0.49**	**0.26**
Donor 2	1.00	0.78	**0.64**	**0.40**	**0.35**
Donor 3	1.00	**0.74**	**0.58**	**0.44**	**0.27**
Donor 4	1.00	0.77	**0.56**	**0.35**	**0.19**
Donor 5	1.00	0.78	**0.60**	**0.38**	**0.24**
Mean	1.00	0.77	**0.62**	**0.41**	**0.26**
C (high ROS)					
Donor 1	1.00	0.78	**0.64**	**0.34**	**0.22**
Donor 2	1.00	0.83	**0.65**	**0.35**	**0.23**
Donor 3	1.00	0.78	**0.56**	**0.29**	**0.07**
Donor 4	1.00	0.77	**0.49**	**0.26**	**0.06**
Donor 5	1.00	0.85	**0.45**	**0.18**	**0.11**
Mean	1.00	0.80	**0.56**	**0.28**	**0.14**

Samples are considered toxic (bold values) if SMI < 0.75 (sperm toxic). Average SMI: Group A (0.54) > Group B (0.41) > Group C (0.28) at 12 h; Group A (0.40) > Group B (0.26) > Group C (0.14) at 24 h.
SMI, sperm motility index.

correlated with both ROS levels in washed sperm and ROS-TAC scores. The ROS levels measured in whole ejaculates accurately reflected *in vivo* OS status; thus, this modified method may overcome the drawbacks of previous methods (6). A small study found high ROS in whole ejaculates was associated with sperm motility (7). In this large study using whole ejaculates, we found that ROS levels were significantly associated with abnormal morphology of sperm, but not motility. The reason for the inconsistent finding with motility is unclear. Further study is needed to replicate the findings.

The HSMA is an important bioassay for testing gamete toxicity, which has been used extensively in assisted reproductive technology laboratories. In this study, the detrimental effects of seminal plasma with different ROS levels were evaluated by HSMA. Using HSMA, we found in the current study that seminal plasma from MFI patients reduced motility of sperm from subjects with normal semen characteristics and the reduction rates increased with increasing ROS levels. This further supports the possible critical role of OS in the development of MFI. Many factors could cause or contribute to ROS levels. For example, it was reported that ROS can be induced by electroshock, and after electrical stimulation, the seminal plasma not only reduced the motility parameters but also decapacitated sperm and prevented fertilization in a number of species, including cattle, pigs, mice, primates, and humans. It is also possible that other factors in semen work synergistically with ROS in the etiology of MFI. Furthermore, we found that ROS levels for donors with normal semen characteristics were similar to that for MFI patients with ROS levels at the lowest 25 percentile. These findings indicate that factors other than OS may play a critical role in the pathogenesis of the disease, among those MFI patients with low ROS levels. This finding is consistent with the fact that ROS at basal low level is essential for normal male reproductive function (4). The findings are significant for multiple reasons. Our finding may provide a possible biological explanation for the inconsistent results in previous clinical trials using antioxidant strategies in MFI patients. Our finding may indicate that antioxidant strategy may only be effective among those with a high level of ROS.

Table 5. Assessing the Interaction Between Time and Group Using Repeated Measurement Approach

Source	*DF*	*Type III SS*	*Mean square*	F-*value*	p-*value*
Group	2	0.28423467	0.14211733	75.57	< 0.0001
Donor	4	0.10179467	0.02544867	13.53	0.0012
Time	4	0.66598630	0.16649658	84.02	< 0.0001
Time × Group	8	0.14472533	0.01809067	9.13	< 0.0001
Time × Donor	16	0.08891200	0.00555700	3.24	0.0023

Due to the important potential of ROS measure in the development of personalized antioxidant strategy and clinical practice, we divided MFI patients into OS-positive and OS-negative groups, based on the critical value of ROS level (*e.g.*, 95 percentile cutpoint of ROS levels among healthy donors). We found that the OS-positive rate was 49.4% among Chinese MFI patients, which was similar to the previous reports conducted in other populations. Many assays have been developed to measure ROS. The chemiluminescence assay (5) used two possible probes: luminol and lucigenin. The luminol assay was more advantageous because it was able to measure H_2O_2, $O_2^{\cdot -}$, and OH^- levels. This was the first study measuring ROS levels among the Chinese population using chemiluminescence assay.

Along with previous association studies among MFI patients, due to the cross-sectional nature of the current study, the temporal sequence was unclear for the association between OS and semen quality. However, in our study, we have evaluated the effect of seminal plasma obtained from MFI patients on sperm donated by subjects with normal semen characteristics. We still cannot exclude the possibility that the observed association with ROS levels may be caused by other factors correlated with ROS levels, such as lipid peroxidation products and inflammation. Further studies are needed to explore this possibility.

In conclusion, our observations lead to the hypothesis that OS plays a critical role in the development of MFI among those with high ROS levels, but not those with low ROS levels. It is possible that factors other than OS may be involved in the pathogenesis of male infertility among those with low levels of ROS levels. Therefore, supplementation of antioxidants may be only helpful among those with high ROS levels. This hypothesis needs to be confirmed in future studies, including personalized clinical trials.

Notes

Subjects

The study was approved by the institutional review board of the Shanghai Institute of Planned Parenthood Research, and all patients provided written informed consent. Included

in the study were 1092 MFI patients. MFI was defined as the inability of a couple to conceive a child after 2 years of unprotected intercourse with a normal female partner or spouse with a normal ovulation (by follicular ultrasonography, luteal phase progesterone levels, and endometrial biopsy), and tubal patency. They were recruited from the Reproductive Hospital affiliated with Shanghai Institute of Planned Parenthood Research and Ruijin Hospital affiliated with Shanghai Jiaotong University, China, between January 2005 and December 2007. Patients with azoospermia were excluded from the current study. Additionally, we recruited 50 donors who had normal semen characteristics according to World Health Organization (WHO) guidelines.

Semen collection and assessment of semen parameters

The semen samples were collected using the method introduced by the WHO. Immediately after liquefaction, fresh sperm concentration and percent motility were assessed using a computer-assisted sperm analysis system (Cyto-S; Alpha Innotech Corp.). The integrity of the sperm membrane (viability) was evaluated using Live/Dead Sperm Viability Kit (L-7011; Invitrogen). For morphological evaluation, seminal smears were stained with Diff-Quik stain and assessed. The abnormal morphology rates, head defect, body/midpiece defect, tail defect, teratozoospermia index (TZI), and SDI were calculated according to WHO guidelines.

Chemiluminescence measurement of ROS level in whole ejaculates

The ROS levels in fresh semen were determined within 2 h of sample collection using luminol (5-amino-2, 3-dihydro-1, 4-phthalazinedione; Sigma) as the probe. First, 4 μl of 25 mM luminol prepared in dimethyl sulfoxide (Sigma) and 8 μl of HRP (12.4 U IV HRP; 310 U/mg) were added to 400 μl of neat semen. ROS levels were determined by measuring chemiluminescence with a Berthold luminometer (model: Sirius C-2). The results were expressed as relative light units per second (RLU/s). After the parameters were measured, the sperm and seminal plasma were separated by centrifugation at $300\times g$ and stored at -80°C.

The effect of frozen MFI seminal plasma with different levels of ROS on motility of fresh sperm

Based on the ROS levels in whole ejaculates, seminal plasma specimens from 1092 MFI patients were classified into three categories: specimens with the ROS level at the lowest 25th percentile, among 25th–75th percentile, and at the highest 75th percentile. Then, 10 frozen specimens were randomly selected from each category. Thus, a total of 30 specimens were selected (10 per group): Group A with a low ROS level, Group B with a medium level, and Group C with a high level. Subsequently, the 10 specimens in the same group were mixed with equal volume and the mixed seminal plasma samples were then centrifuged at $10^5\times g$ at 4°C for 2 h in order to discard any cell segments. Four tissue-culture tubes were prepared. Three of the tubes contained 100 μl HTF (HTF; Irvine Scientific) and 100 μl mixed seminal plasma from Groups A, B, and C, respectively. The fourth tube contained only 200 μl HTF as a control.

Fresh sperm samples were collected from five donors who had normal characteristics according to WHO guidelines. After washing and swimming up, sperm suspension from one donor was added into the above-mentioned four tubes (the final concentration was adjusted to 5×10^6 motile spermatozoa/ml) and cultured for 24 h at 37°C in a 5% CO_2 incubator. Five-microliter samples were collected from each tube at 3, 6, 12, and 24 h for motility detection. The above-mentioned experiment was repeated five times for five different donors.

The SMI was calculated as follows:

$$\mathrm{SMI} = \frac{\text{Percentage of progressive motility in the test sample}}{\text{Percentage of progressive motility in the control}}$$

Samples were regarded as toxic if the motility index was <0.75.

Statistical analysis

The Spearman correlation coefficient was used to examine the associations between ROS levels and semen parameters. Since the distribution of ROS levels was skewed (coefficient of skewness = 11.06), the Wilcoxon signed-rank tests were utilized to compare the sperm parameters between OS-positive and OS-negative groups. Repeated measurement analysis of variance was used to compare SMI values with time in HSMA. p values of <0.05 (two-sided probability) were interpreted as statistically significant. Statistical analyses were conducted using SAS statistical software (version 9.1; SAS Institute).

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Address correspondence to:
Dr. Hui-Juan Shi
Shanghai Institute of Planned Parenthood Research
2140 Xietu Road
Shanghai 200032
People's Republic of China

E-mail: shihuijuan@sippr.stc.sh.cn

Dr. Qi Dai
Institute for Medicine and Public Health
2525 West End Ave.
Sixth Floor, Suite 600
Nashville, TN 37203

E-mail: qi.dai@vanderbilt.edu

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Abbreviations Used

ALH = amplitude of lateral head displacement
BCF = beat cross frequency
HSMA = human sperm motility assay
LIN = linearity
MAD = mean angle of deviation
MFI = male factor infertility
OS = oxidative stress
RLU/s = relative light units per second
ROS = reactive oxygen species
SDI = sperm deformity index
SMI = sperm motility index
STR = straightness
TAC = total antioxidant capacity
TZI = teratozoospermia index
VAP = average path velocity
VCL = curvilinear velocity
VSL = straight-line velocity
WHO = World Health Organization
WOB = wobble

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