Asian Journal of Andrology (2017) 19, 350–354
www.asiaandro.com; www.ajandrology.com

Open Access

ORIGINAL ARTICLE

Semen Analysis

Inverse correlation between reactive oxygen species in unwashed semen and sperm motion parameters as measured by a computer-assisted semen analyzer

Teppei Takeshima[1], Yasushi Yumura[1], Kengo Yasuda[1], Hiroyuki Sanjo[1], Shinnosuke Kuroda[1], Hiroyuki Yamanaka[1], Akira Iwasaki[1,2]

This study investigated the correlation between sperm motion parameters obtained by a computer-assisted semen analyzer and levels of reactive oxygen species in unwashed semen. In total, 847 patients, except for azoospermic patients were investigated. At the time of each patient's first consultation, semen parameters were measured using SMAS™ or CellSoft 3000™, and production of reactive oxygen species was measured using a computer-driven LKB Wallac Luminometer 1251 Analyzer. The patients were divided into two groups: reactive oxygen species - positive and negative. The semen parameters within each group were measured using one of the two computer-assisted semen analyzer systems and then compared. Correlations between reactive oxygen species levels and sperm motion parameters in semen from the reactive oxygen species - positive group were also investigated. Reactive oxygen species were detected in semen samples of 282 cases (33.3%). Sperm concentration ($P < 0.01$; $P < 0.01$), motility ($P < 0.01$; $P < 0.05$), and progressive motility ($P < 0.01$; $P < 0.01$) were markedly lower in the reactive oxygen species - positive group than in the reactive oxygen species - negative group. Among the sperm motion parameters in the reactive oxygen species - positive group, sperm concentration ($P < 0.01$; $P < 0.01$), motility ($P < 0.05$; $P < 0.01$), mALH ($P < 0.05$; $P < 0.01$), and progressive motility ($P < 0.05$; $P < 0.01$) also showed inverse correlations with the logarithmic transformed reactive oxygen species levels. Therefore, this study demonstrated that excessive reactive oxygen species in semen damage sperm concentration, motility, and other sperm motion parameters.

Asian Journal of Andrology (2017) **19**, 350–354; doi: 10.4103/1008-682X.173933; published online: 1 March 2016

Keywords: oxidative stress; reactive oxygen species; semen analysis; sperm motility

INTRODUCTION

Reactive Oxygen Species (ROS) such as hydrogen peroxide (H_2O_2), superoxide anion ($\cdot O_2^-$), and hydroxyl radicals ($\cdot OH$) can be produced by human living spermatozoa incubated under aerobic conditions.[1] In addition, the principal sources of endogenous ROS in semen is considered either seminal leukocytes[2–4] or abnormal spermatozoa.[5–7] Aitken and Clarkson first detected ROS in washed human semen in 1987 using a chemiluminescence method.[2] Since then, there have been many reports about the influence of oxidative stress on male fertility. ROS is important to the normal functions of spermatozoa, such as hyperactivation, capacitation, and acrosome reaction.[8,9] On the other hand, excessive levels of ROS are known to cause significant damage to both mitochondrial and nuclear DNA within human spermatozoa.[10–13] ROS also damage polyunsaturated fatty acids, and in particular, docosahexaenoic acid with six double bonds per molecule, in spermatozoa cellular membranes to generate lipid peroxides.[14] Excessive lipid peroxide production leads to the loss of sperm motility and membrane fluidity for fusion with the vitelline membrane of the oocyte. Iwasaki and Gagnon using the same chemiluminescence method reported that ROS formation in whole semen was detected in 40% of infertile male patients and that ROS levels were inversely correlated with sperm motility and linearity.[15] The Computer-Assisted Semen Analyzer (CASA) can easily analyze sperm concentration, motility, and multiple other motion parameters such as straight-line velocity (VSL), curvilinear velocity (VCL), linearity index (LIN: the ratio of VSL to VCL), beat cross frequency (BCF), mean amplitude of lateral head displacement (mALH), and progressive motility. Although it has been suggested that these sperm motion parameters are very important in the evaluation of male infertility, there have been few reports on the correlation between sperm motion parameters and ROS in semen. Using a chemiluminescence method, we measured ROS levels in whole semen samples from male infertility patients and investigated possible correlations between ROS concentration and sperm motion parameters as measured using CASA.

MATERIALS AND METHODS

Subjects

A total of 847 male patients (mean age: 37 years; range: 21–68 years) consulting at our male infertility clinic at Yokohama City University Medical Center, Reproductive Center from April 1994 to December 2013 were evaluated retrospectively. Azoospermic patients were

[1]Department of Urology, Reproductive Center, Yokohama City University Medical Center, Yokohama, Kanagawa, Japan; [2]YMRAC Urology Office, Yokohama, Kanagawa, Japan.
Correspondence: Dr. T Takeshima (teppeitalia@gmail.com)
Received: 22 July 2015; Revised: 11 October 2015; Accepted: 22 December 2015

excluded. Except for the partners of gynecological patients, every patient received a medical interview, medical examination, and endocrine examination at the first consultation. All patients provided their informed consent for participation. The study design was approved by the Institutional Review Board of Yokohama City University Medical Center.

Semen collection and assessment of semen parameters

Semen specimens were collected by masturbation after 48–120 h of sexual abstinence. Semen analyses were conducted 3 times before treatment with the CellSoft 3000™ (CRYO Resources Ltd., NY, USA) in 288 patients or the Sperm Motility Analyzing System (SMAS™: DITECT Ltd., Tokyo, Japan), a new CASA system in 559 patients that analyzes sperm at 37°C after complete liquefaction. In accordance with the criteria of the World Health Organization (WHO) standards of 2010,[16] the following parameters were measured: semen volume, sperm concentration ($\times 10^6$ ml^{-1}), sperm motility (%), VSL (μm s^{-1}), measured as the straight line distance from beginning to end of a sperm track divided by the time taken; VCL (μm s^{-1}), measured as the total distance traveled by a given sperm divided by the time elapsed; linearity index (LIN, the ratio of VSL to VCL); mALH (μm), measured as the mean width of sperm head oscillation; BCF (Hz), defined as the frequency of the sperm head crossing the sperm average path; and progressive motility (%), or the fraction of spermatozoa that progress at a rate >25 μm s^{-1}.

SMAS™ consists of a digital scanning camera, a personal computer with a digital frame grabber, image-processing software, and a computer monitor. This setup requires just a few minutes to analyze semen and can be performed easily. This method was selected on the basis of the reports from Komori *et al*. and Akashi *et al*. that showed significant correlations between the semen parameters (sperm concentration and motility) obtained using SMAS™ and manual semen analysis,[17,18] and between those using CellSoft 3000™ and manual semen analysis, although the value of sperm motility as measured by SMAS™ and CellSoft 3000™ is known to be slightly lower than that obtained by manual determination.

However, sperm motion parameters such as VSL, VCL, LIN, mALH, BCF, and progressive motility as measured by CASA were calculated on the basis of a unique algorithm on each CASA system. Therefore, the reduction formulas were not established between the CASA systems. Moreover, for statistical neatness, all the parameters were evaluated separately within the datasets generated by each CASA system. To standardize the linearity index, those values measured by CellSoft 3000™ were recalculated as the ratio of VSL divided by VCL as measured by CellSoft 3000™.

ROS measurement

ROS formation levels in unwashed semen were measured using a computer-driven LKB Wallac 1251 Luminometer™ (LKB Wallac, Turku, Finland) simultaneously with the routine semen analysis performed within the first day of consultation. Chemiluminescence was recorded after the addition of 40 μl of 100 mmol l^{-1} luminol (5-amino-2,3-dihydro-1,4-phtalazine-dione) to 500 μl of unwashed semen. ROS formation was considered positive when the luminescence was ≥0.1 mV s^{-1} at peak value.[15] The integrated chemiluminescence between 0 and 30 min after the addition of luminol to unwashed semen was expressed by the values mV per 30 min 10^{-8} spermatozoa, and regarded as a ROS level of the sample (**Figure 1**). Because of the skewed distribution of ROS levels, data were normalized by logarithmic transformation prior to further statistical analyses.

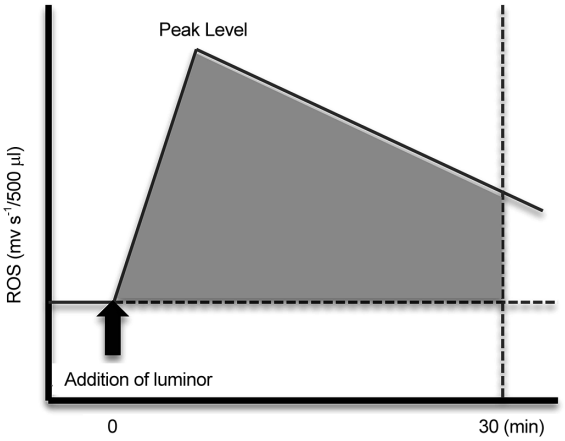

Figure 1: Reactive oxygen species (ROS) measurement by chemiluminescence method. When the peak level was ≥0.1 mv s^{-1}, ROS formation was considered positive. The integral level of ROS production in the present study was calculated by subtraction of the area under the baseline from total chemiluminescence values between 0 and 30 min after the addition of 40 μl of 100 mmol l^{-1} luminol (5-amino-2,3-dihydro-1,4-phtarazine-dione) to 500 μl of unwashed semen, and expressed as the values mV per 30 min 10^{-8} spermatozoa.

Statistical analysis

Statistical analysis was performed using StatMate V™ (ATMS Co., Ltd., Tokyo, Japan). All data are reported as mean ± s.d. values. Patients were divided into two groups: a ROS-positive group and a ROS-negative group. Eight semen parameters were measured using each CASA system and the results were compared between the two groups using the Student's *t*-test. Possible correlation between Log (ROS level) and the eight semen motion parameters in the ROS-positive group was subsequently investigated using Pearson's correlation coefficient. A $P < 0.05$ was considered statistically significant in all cases.

RESULTS

Patients' characteristics and semen parameters are shown in **Table 1**. Patients' primary diseases were grouped as idiopathic infertility, partners of gynecology patients, patients with clinical varicocele, patients after treatment with a cancer drug, and others in decreasing order.

Among the 847 patients, there were 282 cases (33.3%) positive for ROS. Only the semen characteristics of sperm concentration (SMAS: positive group, 30.20 ± 27.71 × 10^6 ml^{-1} *vs* negative group, 36.59 ± 48.87 × 10^6 ml^{-1}, $P < 0.05$; CellSoft: positive group, 12.94 ± 11.57 × 10^6 ml^{-1} *vs* negative group, 40.59 ± 41.96 × 10^6 ml^{-1} $P < 0.01$) and sperm motility (SMAS: positive group, 19.31% ±16.35% *vs* negative group, 23.84% ±17.88%, $P < 0.01$; CellSoft: positive group, 31.84% ±21.93% *vs* negative group, 34.57% ±23.62%, $P < 0.05$) were different between groups. **Figure 2** compares the semen parameters in each group. Sperm concentration ($P < 0.05$; $P < 0.01$), sperm motility ($P < 0.01$; $P < 0.05$), and progressive motility ($P < 0.01$; $P < 0.01$) were lower in the ROS-positive group than in the ROS-negative group in both CASA systems. On the other hand, there were no group differences in VSL ($P = 0.37$; $P < 0.05$), VCL ($P = 0.27$; $P < 0.05$), LIN ($P = 0.06$; $P = 0.06$), mALH ($P = 0.12$; $P = 0.19$), and BCF ($P = 0.26$; $P = 0.20$) with either CASA system.

Figure 3 shows the correlation between the eight sperm motion parameters measured by each CASA system and the values of

Table 1: Patient characteristics and semen parameters in ROS-positive and -negative groups

Category	*Overall*	*ROS*		*Significance*
		Positive	*Negative*	
Patients, *n*	847	282	565	
Age in years, median (range)	37 (21–68)	37 (22–68)	37 (21–54)	
Sperm concentration (×10^6 ml^{-1})				
SMAS (*n*=559)	34.59±43.47	30.20±27.71 (*n*=175)	36.59±48.87 (*n*=384)	*
CellSoft (*n*=288)	33.79±38.77	12.94±11.57 (*n*=113)	40.59±41.96 (*n*=175)	**
Sperm motility (%)				
SMAS (*n*=559)	22.42±17.54	19.31±16.35 (*n*=175)	23.84±17.88 (*n*=384)	**
CellSoft (*n*=288)	33.70±23.13	31.84±21.93 (*n*=113)	34.57±23.62 (*n*=175)	*
Primary disease				
Idiopathic infertility, *n*	366	113	253	
Partner of gynecology patient, *n*	263	66	197	
Clinical varicocele, *n*	114	53	61	
Post anti-cancer drug, *n*	28	13	15	
Sexual dysfunction, *n*	9	4	5	
Other, *n*	67	33	34	

*P<0.05 compared between ROS-positive and -negative group; **P<0.01 compared between ROS-positive and -negative group. ROS: reactive oxygen species

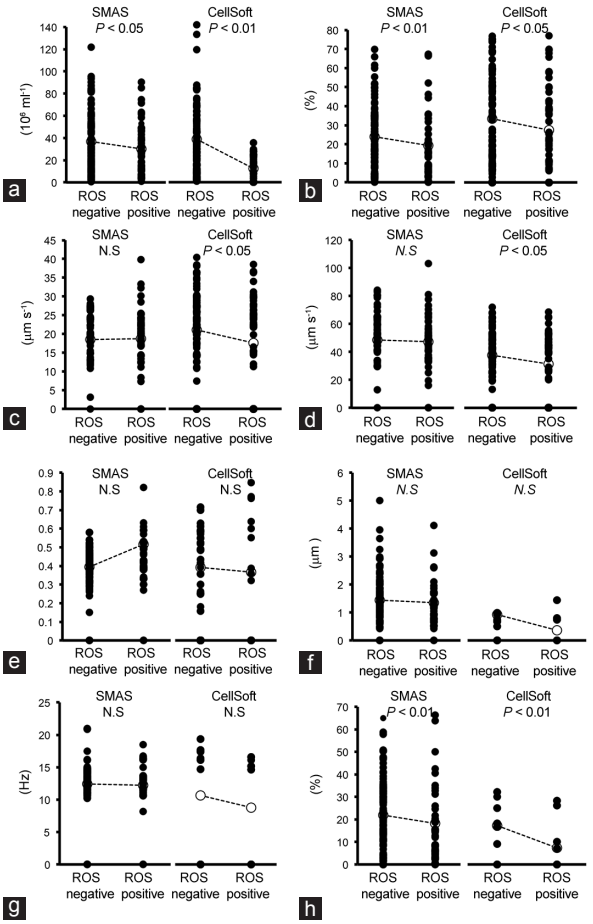

Figure 2: Comparison of the values of semen parameters measured by two CASA systems in ROS-positive and -negative groups. (**a**) Sperm concentration, (**b**) sperm motility, (**c**) straight-line velocity (VSL), (**d**) curvilinear velocity (VCL), (**e**) linearity (LIN), (**f**) mean amplitude of lateral head displacement (mALH), (**g**) beat cross frequency (BCF), (**h**) progressive motility.

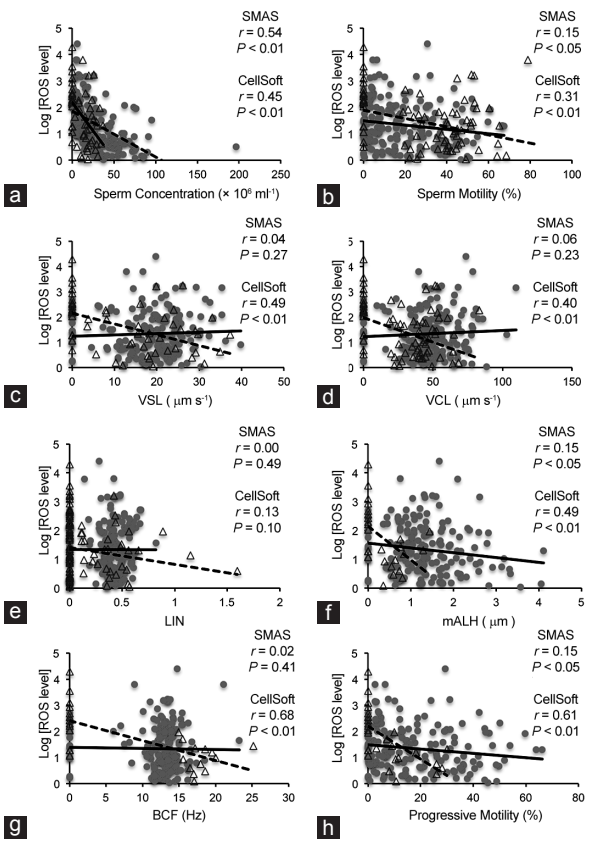

Figure 3: Scatterplot of logarithmic transformed ROS levels, showing each relationship between the ROS levels and sperm motion parameters measured by two CASA systems in ROS-positive group. (**a**) Sperm concentration; (**b**) motility; (**c**) VSL; (**d**) VCL; (**e**) LIN; (**f**) mALH; (**g**) BCF; (**h**) progressive motility. Circle plots expressed SMAS data and approximate lines were continuous. Triangle plots expressed CellSoft data and approximate lines were broken. Sperm concentration ($P < 0.01$; $P < 0.01$), motility ($P < 0.05$; $P < 0.01$), mALH ($P < 0.05$; $P < 0.01$), and progressive motility ($P < 0.05$; $P < 0.01$) showed inverse correlations with ROS level.

logarithmic transformed ROS level in the ROS-positive group. Among the sperm motion parameters, sperm concentration ($P < 0.01$, $r = 0.54$; $P < 0.01$, $r = 0.45$), sperm motility ($P < 0.05$, $r = 0.15$; $P < 0.01$, $r = 0.31$), mALH ($P < 0.05$, $r = 0.15$; $P < 0.01$, $r = 0.49$), and

progressive motility ($P < 0.05$, $r = 0.15$; $P < 0.01$, $r = 0.61$) showed significant inverse correlations with logarithmic transformed ROS in both CASA systems.

DISCUSSION

The spermatozoal plasma membrane is rich in the polyunsaturated fatty acids necessary to maintain the fluidity required for membrane fusion during fertilization, and therefore, it is highly susceptible to oxidative stress.[14,19] The mechanism of ROS-induced declines in sperm motility is explained by the fact that excessive ROS damages spermatozoal membrane polyunsaturated fatty acids and produces lipid peroxides. Moreover, accumulation of lipid peroxides causes degeneration of the spermatozoal membrane, loss of membrane fluidity, a decline in the capacity for sperm-oocyte fusion, and decreased sperm flagellum motion due to lower intracellular sperm adenosine triphosphate levels.[20,21]

In 1987, Aitken and Clarkson first detected ROS in human washed semen using a chemiluminescence method.[2] Since then, the influence of ROS on male fertility has been frequently reported, and now, oxidative stress is believed to be one of the important factors of male idiopathic infertility. Using the same chemiluminescence method, Iwasaki and Gagnon reported that ROS formation in semen was detected in approximately 40% of infertile male patients and was inversely correlated with sperm motility and linearity.[15] They reported that ROS levels in whole semen did not correlate significantly with other sperm motion parameters.[15] Agarwal *et al*. subsequently reported that the ROS level in semen with poor qualities of sperm concentration, motility, and velocity was significantly higher than that of semen with good quality,[22] and that these parameters were related to male fertility. Moreover, there are few reports on the correlation between ROS levels in semen and other sperm motion parameters. We measured these parameters using SMAS™, a new CASA system, and conventional CellSoft 3000™. SMAS™ has been commercially available since 2002 in Japan at approximately one-tenth the cost of a traditional CASA system, despite requiring just a few minutes to analyze multiple sperm motion parameters.

According to our results, ROS detection was positive in 33.3% of unwashed patient semen samples. This finding does not conflict with the results of other published studies. Compared with the ROS-negative group, the ROS-positive group showed a significant decline in sperm concentration, sperm motility, and progressive motility. In addition, the logarithmic transformed ROS levels in the ROS-positive group showed a strong inverse correlation with the sperm concentration, motility, mALH, and progressive motility. Among these parameters, sperm concentration showed the strongest inverse correlation with semen ROS level. This relationship could originate from the large number of immature or abnormal spermatozoa, a prominent ROS source, present in hypospermatogenetic testes producing excessive ROS levels in semen. In addition, because the ROS levels in our data represent the integration of chemiluminescence per one unit or the number of spermatozoa ($\times 10^8$), the fewer spermatozoa that are present, the larger the ROS level per individual unit will be. In other words, low sperm concentration indicates frailty of spermatozoa against the harm of ROS in semen. There have been few reports on the relationship between sperm concentration and ROS in unwashed semen.

Our results also demonstrated that there was a strong negative correlation between the logarithmic transformed ROS level and sperm motility as well as mALH and progressive motility. This mechanism might be explained by spermatozoal membrane damage and depressed sperm flagellum motion as described above. The motility parameters ALH, VSL, VCL, and LIN have been reported to be correlated with fertility by several authors.[23–27] In our study, VSL and VCL measured using SMAS did not show correlation with ROS level, but ALH had strong correlation with ROS level. Based on the large number of samples (total 847 cases, 282 positive cases) evaluated, our investigation strongly supports the hypothesis that excessive ROS formation in semen may contribute to decrease in sperm motility and male fertility.

However, we found no correlation between ROS in semen and the linearity index (LIN) in both CASA systems, which contradicts previous reports. This difference may be explained in part as described below. The value of LIN previously reported indicates that spermatozoal straight speed was expressed using a grade of 10 steps. On the other hand, LIN obtained by SMAS™ represents the ratio of VSL to VCL. Moreover, to standardize the LIN values, those measured by CellSoft 3000™ were recalculated as the ratio of VSL divided by VCL as measured by CellSoft 3000™ in this study. Because of these differences, the value of LIN in this study may not correlate with ROS level, even though VSL and VCL values measured by CellSoft 3000™ were correlated. Moreover, our results demonstrated discrepancy between each CASA systems. It might be explained by the fact that each CASA system had specific measurement algorithms. Further investigation is required to better explain this difference.

Our investigation indicated that excessive ROS in semen was largely responsible for the loss of male fertility. However, in 2009, Yumura *et al*. reported the cut-off value of ROS in semen for retaining fertility as 4.35 mV per 30 min 10^{-6} sperm as obtained using a Luminometer,[28] and patients with a value above this threshold obtained significantly lower pregnancy rates. We have not yet attempted to validate the accuracy of this cut-off value. However, look forward to being able to report a positive outcome from this study in the near future.

Finally, as decreasing ROS levels in semen may bring considerably improved sperm motility and will protect sperm from DNA damage and fragmentation, ultimately improving male fertility, it is important to determine the most effective drugs for reducing ROS formation in semen. Although there are a few reports on the efficacy of antioxidant treatments, such as Vitamin C, Vitamin E, coenzyme Q10, and so on for improving male fertility and sperm motility;[11,29,30] large, randomized, controlled studies are required and should be established for examining the efficacy.

CONCLUSIONS

Our investigation demonstrated that high levels of ROS in human semen decrease sperm concentration in semen and sperm motility along with other sperm motion parameters. Excessive ROS in semen was also detected in a certain percentage in patients with or without a diagnosed primary disease. These data suggest that ROS formation may be one of the causes of idiopathic infertility and may also be caused directly by degenerated spermatogenetic cells.

AUTHOR CONTRIBUTIONS

TT, YY, and AI conceived and designed the study; TT, YY, KY, HS, SK, and HY performed the experiments; TT reviewed and analyzed the data and drafted the manuscript; YY and AI supervised the study and gave final approval of the version to be submitted.

COMPETING INTERESTS

All authors declare no competing financial interests.

ACKNOWLEDGMENTS

This study was supported by grants from Strategic Research Promotion of Yokohama City University (Grant No. IR2502). We thank Dr. Kazumi Noguchi for supervising this study.

REFERENCES

1 Holland MK, Alvarez JG, Storey BT. Production of superoxide and activity of superoxide dismutase in rabbit epididymal spermatozoa. *Biol Reprod* 1982; 27: 1109.
2 Aitken RJ, Clarkson JS. Cellular basis of defective sperm function and its association with the genesis of reactive oxygen species by human spermatozoa. *J Reprod Fertil* 1987; 81: 459–69.
3 Aitken RJ, Clarkson JS. Significance of reactive oxygen species and antioxidants in defining the efficacy of sperm preparation techniques. *J Androl* 1988; 9: 367–6.
4 Sharma RK, Pasqualotto AE, Nelson DR, Thomas AJ Jr., Agarwal A. Relationship between seminal white blood cell counts and oxidative stress in men treated at an infertility clinic. *J Androl* 2001; 22: 575–83.
5 Gomez E, Buckingham DW, Brindle J, Lanzafame F, Irvine DS, *et al*. Development of an image analysis system to monitor the retention of residual cytoplasm by human spermatozoa: correlation with biochemical markers of the cytoplasmic space, oxidative stress, and sperm function. *J Androl* 1996; 17: 276–87.
6 Sakkas D, Seli E, Bizzaro D, Tarozzi N, Manicardi GC. Abnormal spermatozoa in the ejaculate: abortive apoptosis and faulty nuclear remodeling during spermatogenesis. *Reprod Biomed Online* 2003; 7: 428–32.
7 Koppers AJ, Garg ML, Aitken RJ. Stimulation of mitochondrial reactive oxygen species production by unesterified, unsaturated fatty acids in defective human spermatozoa. *Free Radic Biol Med* 2010; 48: 112–9.
8 Sharma RK, Agarwal A. Reactive oxygen species and male infertility. *Urology* 1996; 14: 835–50.
9 Aitken RJ. Molecular mechanisms regulating sperm function. *Mol Hum Reprod* 1997; 3: 169–73.
10 Aitken RJ, Gordon E, Harkiss D, Twigg JP, Milne P, *et al*. Relative impact of oxidative stress on the functional competence and genomic integrity of human spermatozoa. *Biol Reprod* 1998; 59: 1037–46.
11 Kodama H, Yamaguchi R, Fukuda J, Kasai H, Tanaka T. Increased oxidative deoxyribonucleic acid damage in the spermatozoa of infertile men. *Fertil Steril* 1997; 68: 519–24.
12 Sawyer DE. Analysis of gene-specific DNA damage and single-strand DNA breaks induced by pro-oxidant treatment of human spermatozoa *in vitro*. *Mutat Res* 2003; 529: 21–34.
13 Santiso R, Tamayo M, Gosalvez J, Meseguer M, Garrido N, *et al*. Simultaneous determination *in situ* of DNA fragmentation and 9-oxoguanine in human sperm. *Fertil Steril* 2010; 93: 314–8.
14 Jones R, Mann T, Sherins RJ. Peroxidative breakdown of phospholipids in human spermatozoa, spermicidal effects of fatty acid peroxides and protective action of seminal plasma. *Fertil Steril* 1979; 31: 531–7.
15 Iwasaki A, Gagnon C. Formation of reactive oxygen species in spermatozoa of infertile patients. *Fertil Steril* 1992; 57: 409–16.
16 World Health Organization. WHO Laboratory Manual for the Examination and Processing of Human Semen. 5th ed. Geneva, Switzerland: World Health Organization; 2010. p. 21–36.
17 Komori K, Tsujimura A, Ishijima S, Tanjapatkul P, Fujita K, *et al*. Comparative study of sperm motility analysis system and conventional microscopic semen analysis. *Reprod Med Biol* 2006; 5: 195–200.
18 Akashi T, Watanabe A, Komiya A, Fuse H. Evaluation of the sperm motility analyzer system (SMAS) for the assessment of sperm quality in infertile men. *Syst Biol Reprod Med* 2010; 56: 473–7.
19 Alvarez JG, Touchstone JC, Blasco L, Storey BT. Spontaneous lipid peroxidation and production of hydrogen peroxide and superoxide in human spermatozoa – Superoxide dismutase as major enzyme protectant against oxygen toxicity. *J Androl* 1987; 8: 338–48.
20 De Lamirande E, Gagnon C. Reactive oxygen species and human spermatozoa II. Depletion of adenosine triphosphate plays an important role on the inhibition of sperm motility. *J Androl* 1992; 13: 379–86.
21 Kovalski NN, De Lamirande E, Gagnon C. Reactive oxygen species generated by human neutrophils inhibit sperm motility: protect effect on seminal plasma and scavengers. *Fertil Steril* 1992; 58: 809–16.
22 Agarwal A, Ikemoto I, Loughlin KR. Relationship of sperm parameters to levels of reactive oxygen species in semen specimens. *J Urol* 1994; 152: 107–10.
23 Aitken RJ, Best FS, Warner P, Templeton A. A prospective study of the relationship between semen quality and fertility in cases of unexplained infertility. *J Androl* 1984; 5: 297–303.
24 Barratt CL, Tomlinson MJ, Cooke ID. Prognostic significance of computerized motility analysis for *in vivo* fertility. *Fertil Steril* 1993; 60: 520–5.
25 Irvine DS, Macleod IC, Templeton AA, Masterton A, Taylor A. A prospective clinical study of the relationship between the computer-assisted assessment of human semen quality and the achievement of pregnancy *in vivo*. *Hum Reprod* 1994; 9: 2324–34.
26 Paston MJ, Sarkar S, Oates RP, Badawy SZ. Computer-aided semen analysis variables as predictors of male fertility potential. *Arch Androl* 1994; 33: 93–9.
27 Krause W. Computer-assisted semen analysis systems: comparison with routine evaluation and prognostic value in male fertility and assisted reproduction. *Hum Reprod* 1995; 10: 60–6.
28 Yumura Y, Iwasaki A, Saito K, Ogawa T, Hirokawa M. Effect of reactive oxygen species in semen on the pregnancy of infertile couples. *Int J Urol* 2009; 16: 202–7.
29 Rolf C, Cooper TG, Yeung CH, Nieschlag E. Antioxidant treatment of patients with asthenozoospermia or oligoasthenozoospermia with high-dose vitamin C and Vitamin E: a randomized, placebo-controlled, double-blind study. *Hum Reprod* 1999; 14: 1028–33.
30 Balercia G, Buldreghini E, Vigini A, Tiano L, Paggi F, *et al*. Coenzyme Q10 treatment in infertile men with idiopathic asthenozoospermia: a placebo-controlled, double blind randomized trial. *Fertil Steril* 2009; 91: 1785–92.