Asian Journal of Andrology (2010) 12: 271–277

www.nature.com/aja

Original Article

Comparative study of the effects of three semen preparation media on semen analysis, DNA damage and protamine deficiency, and the correlation between DNA integrity and sperm parameters

Charoenchai Chiamchanya[1], Nattpawit Kaewnoonual[2], Pachara Visutakul[3], Sirikul Manochantr[3], Jirattikan Chaiya[4]

[1]*Department of Obstetric and Gynecology, Faculty of Medicine, Thammasat University, Pathumthani, Bangkok 12120, Thailand*
[2]*Faculty of Medicine, Thammasat University, Pathumthani, Bangkok 12120, Thailand*
[3]*Faculty of Medicine, Office of Graduate Studies, Thammasat University, Pathumthani, Bangkok 12120, Thailand*
[4]*Infertility Unit, Thammasat University Hospital, Pathumthani, Bangkok 12120, Thailand*

Abstract

Semen samples collected from 28 male partners of infertile couples were divided into three equal aliquots and prepared with three selected media, such as PureSperm® (Nidacon, Gothenburg, Sweden), Sil-Select Plus™ (Fertipro, Beernem, Belgium) and SpermGrad™ (Vitrolife, Gothenburg, Sweden). The differences in mean percentages of semen parameters were assessed by repeated measures analysis. Correlations of sperm DNA damage, as measured by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, and of protamine deficiency, as measured by chromomycin A3 (CMA3) staining with sperm parameters, were determined by Pearson's correlation. After preparation with all three media, sperm concentrations decreased ($P < 0.05$) while percentages of sperm with normal morphology increased ($P < 0.05$). Percentages of sperm motility, rapid motility and progressive motile concentration (PMC) increased ($P < 0.05$) for each of these parameters, PureSperm preparation gave the best results ($P < 0.05$). The percentage of DNA damage decreased in the PureSperm and Sil-Select Plus preparations (17.9% and 31.3%, respectively, $P < 0.05$) and increased in the SpermGrad preparation (56.3%, $P < 0.05$). Protamine deficiency also decreased in all three kinds of media, 59.3%, 47.7% and 40.3% for PureSperm, Sil-Select Plus and SpermGrad preparations, respectively ($P < 0.05$). The percentage of DNA-damaged sperm was negatively correlated with the percentages of sperm motility, rapid motility and PMC, but was positively correlated with static motility ($P < 0.05$). This comparative study and correlation analysis revealed that PureSperm preparation yielded sperm with the best motility and the lowest percentage of protamine deficiency. The Sil-Select Plus preparation yielded sperm with the lowest amount of DNA damage. The SpermGrad preparation had a high percentage of sperm with normal morphology, but also had the highest percentage of sperm with DNA damage. Sperm DNA damage was correlated with percentages of sperm motility, rapid motility, static motility and PMC.

Asian Journal of Andrology (2010) 12: 271–277. doi: 10.1038/aja.2009.60; published online 14 December 2009.

Keywords: semen parameters, sperm DNA damage, sperm preparation media, sperm protamine deficiency

Correspondence to: Dr Charoenchai Chiamchanya, Department of Obstetric and Gynecology, Faculty of Medicine, Thammasat University, Pathumthani, Bangkok 12120, Thailand.
Fax: +66-2926-9162 E-mail: charoenchai12@hotmail.com
Received: 1 April 2009 Revised: 5 May 2009
Accepted: 20 August 2009 Published online: 14 December 2009

1 Introduction

It is well known that semen quality is a significant factor for determining the success of pregnancy, apart from oocyte and endometrial quality. A semen prepa-

ration medium that results in good semen parameter yields is also an important factor in the treatment of infertility. Routine semen analysis parameters have been used to predict good yield results. However, genetically abnormal sperm may appear motile and morphologically normal [1].

A positive relationship between poor sperm parameters and DNA damage in spermatozoa points to inherent spermatogenesis problems in specific patients [2]. The mechanisms of DNA damage in human spermatozoa include defective sperm chromatin packing, apoptosis and oxidative stress [3–5]. Protamine has a critical role in spermatid differentiation; protamine deficiency can lead to sperm DNA damage and embryonic death in mice [6]. Functionally, it appears that protamines are required for zona pellucida binding and penetration abilities [7].

To obtain the largest number of sperms with normal morphology and the highest percentage of motility while avoiding toxic effects, efficient sperm preparation is required for infertility treatment. Percoll™ gradient density (Kabi Pharmacia, Uppsala, Sweden) has been used effectively, and has been approved for sperm selection since 1988 [8–11] owing to its superior selection ability compared with simple washing or swim-up preparations [12–15]. However, several preparation media that use centrifugation gradients have been studied [8–11], including Isolate™, SpermGrad™, Sil-Select Plus™, Optiprep™ and PureSperm®. Among them, PureSperm yielded similar sperm parameters to those of Percoll and could be considered a suitable substitute for Percoll, which was abandoned in 1996 owing to toxicity [16]. On the other hand, SpermGrad and Sil-Select Plus had average parameter values, and Optiprep had poor sperm concentration values. Although Isolate had optimal concentration values, its selection was unsatisfactory, because it produced a large number of immotile sperm [17]. PureSperm was reported to yield better sperm concentrations when compared with Isolate or the swim-up method [18].

The first aim of this study was to compare the effects of three selected semen preparation media, PureSperm (Nidacon, Gothenburg, Sweden), Sil-Select Plus (Fertipro, Beernem, Belgium) and SpermGrad (Vitrolife, Gothenburg, Sweden), on semen analysis parameters, DNA damage and protamine deficiency in postpreparation spermatozoa. The second aim was to determine the correlations between semen analysis parameters and sperm DNA integrity (DNA damage and protamine deficiency) in postpreparation samples.

2 Materials and methods

This prospective study was performed in 28 males from infertile couples who attended the fertility clinic at Thammasat University Hospital (Bangkok, Thailand) in 2007. Semen analysis was processed according to World Health Organization (WHO) [19] and Kruger strict criteria [20]. The semen samples were divided into three equal aliquots and prepared with three sperm preparation media (PureSperm, Sil-Select Plus and SpermGrad). Sperm analysis parameters of the three preparations were compared. Sperm concentration, motility and progressive motile concentration (PMC), which is defined as all sperm having an average pathway velocity higher than the medium velocity and having a straightness of more than 70%, were measured by computer-aided sperm analysis. Sperm morphology was assessed by Papanicolaou staining, based on Kruger strict criteria [20]. Evaluation of DNA damage by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay and evaluation of protamine deficiency by chromomycin A3 (CMA3) staining were also performed. On the basis of the data from previous studies [16], a calculated sample size of 28 men from infertile couples was included in this study. Analysis of differences in the mean percentages of each sperm parameter for all three media was performed by repeated measures. Pearson's correlation was used to determine correlations between semen parameters and sperm DNA integrity. Statistical significance was set at $P < 0.05$.

3 Results

The results of the comparative study of sperm parameters, DNA damage and protamine deficiency between pre- and postpreparation semen samples are presented in Table 1. The sperm parameters obtained with all three media were the followings: the sperm concentration was decreased ($P < 0.05$) but the percentages of sperm motility, PMC and rapid motility were greatly increased ($P < 0.05$), where the percentages of slow motility were decreased ($P < 0.05$), the percentages of normal sperm morphology were also increased ($P < 0.05$) where the percentages of sperm head defects were decreased ($P < 0.05$). The percentages of DNA-damage sperm decreased after preparation with PureSperm and Sil-Select Plus ($P < 0.05$) but increased after preparation with SpermGrade ($P < 0.05$). The percentages of protamine deficiency decreased after preparation with all three media ($P < 0.05$).

A significant positive correlation was found between the percentage of DNA damage and the percentages of slow motility sperm and tail defect sperm of pre-preparation semen ($P < 0.05$). A significant negative correlation was found between the percentage of protamine-deficient sperm and the percentage of rapid motility sperm of pre-preparation semen ($P < 0.05$). These correlations are shown in Table 2.

A comparison of mean sperm parameter values and of sperm DNA integrity for each postpreparation media are shown in Table 3; no significant difference in sperm concentration was found for any of the three media. By contrast, significant differences ($P < 0.05$) in the mean percentages of sperm motility, rapid motility and PMC were observed among the three media; PureSperm had the highest values (79.0%, 77.2% and 71.0%, respectively), followed by SpermGrad (69.8%, 69.4% and 65.9%, respectively) and Sil-Select Plus (64.2%, 62.5% and 58.8%, respectively). Percentages of sperm with slow motility after PureSperm and SpermGrad preparations were lower when compared with that after Sil-Select Plus preparation (10.5%, 11.2% and 18.5%, respectively; $P < 0.05$). The PureSperm preparation had the lowest percentage of sperm with static motility (10.6%, 17.4% and 18.5%, respectively; $P < 0.05$). Percentages of sperm with normal morphology and head defects obtained with the SpermGrad preparation were found to be higher and lower, respectively, than those obtained with Sil-Select Pluspreparation (44.3% *vs.* 40.9% and 46.5% *vs.* 51.5%, respectively; $P < 0.05$). These two parameters were not significantly different for the PureSperm preparation from those of the other two media.

The Sil-Select Plus preparation gave the lowest percentage of DNA damage, followed by the PureSperm and SpermGrad preparations (9.5%, 11.3% and 21.5%, respectively; $P < 0.05$). The PureSperm preparation had the lowest value of protamine deficiency, followed by the Sil-Select Plus and SpermGrad preparations (9.0%, 11.6% and 13.2%, respectively; $P < 0.05$).

Tables 4, 5 and 6 show the correlations between DNA integrity and semen parameters for all postpreparation media. The percentage of DNA-damaged sperm was negatively correlated with the percentages of sperm motility, rapid motility and PMC ($P < 0.05$), and positively correlated with the percentage of static motility sperm ($P < 0.05$).

4 Discussion

When the results of all three sets of postpreparation semen samples were compared, the percentages of sperm motility, rapid motility and PMC obtained with PureSperm preparation were the highest, followed by those with SpermGrad and Sil-Select Plus preparations. The percentages of slow motility sperm after PureSperm and SpermGrad preparations were lower than that

Table 1. Results of semen analysis, TUNEL assay and CMA3 staining of pre- and postpreparation samples.

Semen analysis parameters	Prewashed (Mean ± SE)	Post-washed					
		PureSperm		Sil-Select Plus		SpermGrad	
		(Mean ± SE)	% Relative change	(Mean ± SE)	% Relative change	(Mean ± SE)	% Relative change
Concentration (million mL^{-1})	45.8 ± 3.5	22.4 ± 4.3	↓51.1*	27.6 ± 6.4	↓39.6*	21.3 ± 5.5	↓53.5*
Motility (%)	50.2 ± 2.8	79.0 ± 2.7	↑57.5*	64.2 ± 3.4	↑27.9*	69.8 ± 3.0	↑39.1*
PMC (%)	37.0 ± 2.3	71.0 ± 2.3	↑91.9*	58.8 ± 3.2	↑59.0*	65.9 ± 2.8	↑78.2*
Rapid motility (%)	48.3 ± 2.8	77.2 ± 2.7	↑59.5*	62.5 ± 3.5	↑29.5*	69.4 ± 2.9	↑43.8*
Slow motility (%)	34.5 ± 2.3	10.5 ± 1.4	↓69.7*	18.5 ± 2.2	↓46.5*	11.2 ± 1.3	↓67.5*
Static motility (%)	15.4 ± 2.4	10.6 ± 2.3	↓30.7*	17.4 ± 3.3	↑13.2	18.5 ± 3.4	↑20.7
Normal morphology (%)	12.8 ± 2.3	43.8 ± 5.8	↑243.9*	40.9 ± 5.3	↑220.0*	44.2 ± 5.2	↑246.2*
Head defect (%)	72.7 ± 2.2	48.5 ± 4.9	↓33.4*	51.5 ± 4.7	↓29.2*	46.5 ± 4.4	↓36.1*
Neck defect (%)	5.2 ± 0.9	2.8 ± 0.4	↓45.9*	3.9 ± 0.9	↓25.3	3.6 ± 0.8	↓30.7
Tail defect (%)	7.0 ± 1.2	4.4 ± 1.2	↓36.4*	4.4 ± 1.1	↓36.4*	5.4 ± 1.3	↓22.0
TUNEL$^+$ (%)	13.8 ± 1.3	11.3 ± 1.6	↓17.9*	9.5± 1.3	↓31.3*	21.5 ± 2.8	↑56.3*
CMA3$^+$ (%)	22.1 ± 1.7	9.0 ± 0.8	↓59.3*	11.6 ± 0.9	↓47.7*	13.2 ± 1.0	↓40.3*

Abbreviation: PMC, progressive motile concentration. TUNEL$^+$ indicates DNA damage; CMA3$^+$ indicates protamine deficiency.
*$P < 0.05$, compared with the corresponding pre-preparation parameters.

Table 2. Correlation between TUNEL assay, CMA3 staining and semen analysis parameters of pre-preparation semen samples.

Parameters	Prewashed (Mean ± SE)	$TUNEL^+$ (13.8 % ± 1.3%)		$CMA3^+$ (22.1% ± 1.7%)	
		Pearson's correlation	*P*-value	Pearson's correlation	*P*-value
Concentration (million mL^{-1})	45.8 ± 3.5	–0.099	0.616	–0.269	0.166
Motility (%)	50.2 ± 2.8	–0.307	0.112	–0.372	0.051
PMC (%)	37.0 ± 2.3	–0.136	0.489	–0.301	0.119
Rapid motility (%)	48.3 ± 2.8	–0.293	0.130	–0.382	0.045
Slow motility (%)	34.5 ± 2.3	0.455	0.015	0.281	0.147
Static motility (%)	15.4 ± 2.4	–0.077	0.696	0.173	0.379
Normal morphology (%)	12.8 ± 2.3	–0.213	0.278	–0.303	0.117
Head defect (%)	72.7 ± 2.2	–0.001	0.997	0.168	0.393
Neck defect (%)	5.2 ± 0.9	–0.169	0.390	0.178	0.366
Tail defect (%)	7.0 ± 1.2	0.408	0.031	0.010	0.961

Abbreviation: PMC, progressive motile concentration. $TUNEL^+$ indicates DNA damage; $CMA3^+$ indicates protamine deficiency.

Table 3. Comparison of mean values of semen parameters, TUNEL assay and CMA3 staining of the three postmedia preparations.

Parameters	PureSperm *vs.* Sil-Select Plus	*P*-value	PureSperm *vs.* SpermGrad	*P*-value	Sil-Select Plus *vs.* SpermGrad	*P*-value
Concentration (million mL^{-1})	22.4 *vs.* 27.6	0.068	22.4 *vs.* 21.3	0.733	27.6 *vs.* 21.3	0.068
Motility (%)	79.0 *vs.* 64.2	0.000	79.0 *vs.* 69.8	0.000	64.2 *vs.* 69.8	0.011
PMC (%)	71.0 *vs.* 58.8	0.001	71.0 *vs.* 65.9	0.005	58.8 *vs.* 65.9	0.001
Rapid motility (%)	77.2 *vs.* 62.5	0.000	77.2 *vs.* 69.4	0.000	62.5 *vs.* 69.4	0.002
Slow motility (%)	10.5 *vs.* 18.5	0.002	10.5 *vs.*11.2	0.643	18.5 *vs.* 11.2	0.004
Static motility (%)	10.6 *vs.* 17.4	0.014	10.6 *vs.* 18.5	0.005	17.4 *vs.* 18.5	0.637
Normal morphology (%)	43.8 *vs.* 40.9	0.161	43.8 *vs.* 44.3	0.810	40.9 *vs.* 44.3	0.034
Head defect (%)	48.5 *vs.* 51.5	0.198	48.5 *vs.* 46.5	0.207	51.5 *vs.* 46.5	0.006
Neck defect (%)	2.8 *vs.* 3.9	0.128	2.8 *vs.* 3.6	0.165	3.9 *vs.* 3.6	0.667
Tail defect (%)	4.4 *vs.* 4.4	1.000	4.4 *vs.* 5.4	0.287	4.4 *vs.* 5.4	0.255
$TUNEL^+$ (%)	11.3 *vs.* 9.5	0.029	11.3 *vs.* 21.5	0.000	9.5 *vs.* 21.5	0.000
$CMA3^+$ (%)	9.0 *vs.* 11.6	0.002	9.0 *vs.* 13.2	0.000	11.6 *vs.* 13.2	0.025

Abbreviation: PMC, progressive motile concentration. $TUNEL^+$ indicates DNA damage; $CMA3^+$ indicates protamine deficiency.

after Sil-Select Plus preparation, whereas the percentage of static motility sperm after PureSperm preparation was lower than that after Sil-Select Plus or SpermGrad preparations. The percentages of normal morphology sperm and sperm head defects obtained with SpermGrad preparation were higher than those after Sil-Select Plus preparation, but were comparable with those of PureSperm preparation. When the DNA integrity of postpreparation semen samples was compared, the percentages of DNA-damaged sperm from the Sil-Select Plus and PureSperm preparations were decreased (the Sil-Select Plus preparation yielded the lowest value), but the percentage of DNA-damaged sperm from the SpermGrad preparation was increased. The percentages of protamine-deficient sperm for all three media preparations were decreased; the PureSperm preparation had the lowest percentage, followed by the Sil-Select Plus and SpermGrad preparations.

The results obtained in this study, when compared with those of other studies [16, 21], revealed that PureSperm preparation yielded the best sperm motility, rapid motility and PMC, as well as the lowest percentage of protamine-deficient sperm. Sil-Select Plus preparation gave the lowest amount of sperm DNA damage, but also resulted in a low percentage of motile sperm. The SpermGrad preparation gave the highest percentage of DNA-damaged sperm, a higher percentage of normal morphology sperm and fewer sperm with head defects

Table 4. Correlation between TUNEL assay, CMA3 staining and semen analysis parameters of PureSperm postpreparation semen samples.

Parameters	PureSperm (Mean ± SE)	TUNEL$^+$ (11.3 % ± 1.6%)		CMA3$^+$ (9.0% ± 0.8%)	
		Pearson's correlation	*P*-value	Pearson's correlation	*P*-value
Concentration (million mL^{-1})	22.4 ± 4.3	–0.331	0.086	–0.113	0.565
Motility (%)	79.0 ± 2.7	–0.590	0.001	–0.064	0.745
PMC (%)	71.0 ± 2.3	–0.493	0.008	0.039	0.842
Rapid motility (%)	77.2 ± 2.7	–0.577	0.001	–0.101	0.609
Slow motility (%)	10.5 ± 1.4	0.103	0.602	0.101	0.609
Static motility (%)	10.7 ± 2.3	0.637	0.000	0.009	0.962
Normal morphology (%)	43.8 ± 5.8	–0.277	0.151	–0.056	0.778
Head defect (%)	48.5 ± 4.9	0.211	0.276	0.035	0.859
Neck defect (%)	2.8 ± 0.4	0.132	0.502	0.373	0.050
Tail defect (%)	4.4 ± 1.2	0.317	0.100	–0.083	0.674

Abbreviation: PMC, progressive motile concentration. TUNEL$^+$ indicates DNA damage; CMA3$^+$ indicates protamine deficiency.

Table 5. Correlation between TUNEL assay, CMA3 staining and semen analysis parameters of Sil-Select Plus postpreparation semen samples.

Parameters	Sil-Select Plus (Mean ± SE)	TUNEL$^+$ (9.5% ± 1.3%)		CMA3$^+$ (11.6% ± 0.9%)	
		Pearson's correlation	*P*-value	Pearson's correlation	*P*-value
Concentration (million mL^{-1})	27.6 ± 6.4	–0.467	0.012	–0.201	0.304
Motility (%)	64.2 ± 3.4	–0.680	0.000	–0.060	0.761
PMC (%)	58.8 ± 3.2	–0.575	0.001	0.010	0.960
Rapid motility (%)	62.5 ± 3.5	–0.675	0.000	–0.075	0.705
Slow motility (%)	18.5 ± 2.2	0.116	0.559	0.276	0.156
Static motility (%)	17.4 ± 3.3	0.624	0.000	–0.125	0.526
Normal morphology (%)	40.9 ± 5.3	–0.275	0.156	0.157	0.424
Head defect (%)	51.5 ± 4.7	0.169	0.390	–0.158	0.422
Neck defect (%)	3.9 ± 0.9	0.058	0.770	–0.035	0.858
Tail defect (%)	4.4 ± 1.1	0.608	0.001	–0.078	0.695

Abbreviation: PMC, progressive motile concentration. TUNEL$^+$ indicates DNA damage; CMA3$^+$ indicates protamine deficiency.

Table 6. Correlation between TUNEL assay, CMA3 staining and semen analysis parameters of SpermGrad postpreparation semen samples.

Parameters	SpermGrad (Mean ± SE)	TUNEL$^+$ (21.5% ± 2.8%)		CMA3$^+$ (13.2% ± 1.0%)	
		Pearson's correlation	*P*-value	Pearson's correlation	*P*-value
Concentration (million mL^{-1})	21.3 ± 5.5	–0.415	0.028	0.064	0.746
Motility (%)	69.8 ± 3.0	–0.661	0.000	0.117	0.552
PMC (%)	65.9 ± 2.8	–0.566	0.002	0.039	0.844
Rapid motility (%)	69.4 ± 2.9	–0.646	0.000	0.119	0.547
Slow motility (%)	11.2 ± 1.3	0.109	0.581	–0.132	0.502
Static motility (%)	18.5 ± 3.4	0.546	0.003	–0.068	0.731
Normal morphology (%)	44.2 ± 5.2	–0.592	0.001	–0.152	0.439
Head defect (%)	46.5 ± 4.4	0.415	0.028	0.141	0.473
Neck defect (%)	3.6 ± 0.8	0.310	0.108	0.126	0.524
Tail defect (%)	5.4 ± 1.3	0.814	0.000	0.049	0.803

Abbreviation: PMC, progressive motile concentration. TUNEL$^+$ indicates DNA damage; CMA3$^+$ indicates protamine deficiency.

than did Sil-Select Plus preparation, but with values comparable with those of PureSperm preparation.

The three preparation media yielded different results, hence the clinical applications of these results should be considered according to the methods of infertility treatment, the results of sperm parameters and the DNA integrity after sperm preparation. The effect of gradient centrifugation on the percentage of sperm with DNA impairment may or may not have an impact on assisted reproduction outcomes; possible effects need to be studied further in future clinical trials.

The negative correlation found in this study between sperm motility, rapid motility and PMC with sperm DNA damage after preparation with all three media were similar to those of a recent study, which reported that early apoptotic sperm numbers were negatively correlated with sperm motility [22]. This finding may be used in a clinical setting to indirectly evaluate sperm DNA quality by measuring sperm motility yields. For example, when we want to select good-quality sperm for intracytoplasmic sperm injection, we may also select the sperm with the highest motility, apart from normal morphology, as high motility correlates with the lowest amount of DNA damage. However, such a correlation could not be applied to prewashed semen or across each postpreparation semen sample with a different preparation medium, because each medium gave different and contrasting yields. For instance, the PureSperm preparation resulted in the best sperm motility, but with higher levels of sperm DNA-damage than Sil-Select Plus preparation, whereas Sil-Select Plus preparation had the smallest effect on increasing sperm motility, but yielded sperm with the highest percentage of normal DNA. SpermGrad preparation had fair sperm motility, but had the worst effect on sperm DNA. This discrepancy may be due to the unique chemical composition of each medium.

5 Conclusion

PureSperm preparation yielded the best sperm motility, rapid motility and PMC, with the lowest percentage of protamine-deficient sperm and a low percentage of DNA-damaged sperm. Sil-Select Plus yielded sperm with the lowest amount of DNA damage, but yielded a low percentage of motile sperm. SpermGrad preparation yielded the highest percentage of DNA-damaged sperm, but had better normal sperm morphology with fewer sperm head defects than those of the Sil-Select Plus preparation. The percentages of sperm motility, PMC, rapid motility and static motility correlated significantly with sperm DNA damage and may be used to predict the DNA quality of sperm from the postpreparation semen samples prepared with each medium.

Acknowledgment

This project was supported by research funds with the approval of the Ethical Committee of the Faculty of Medicine, Thammasat University, Bangkok, Thailand. We thank Assistant Professor, Dr. Junya Pattaraachachai for statistical advice, and all staff members of the Fertility Clinic at the Thammasat University Hospital for laboratory services. We would also like to cordially thank Prof. Kovit Pattanapanyasat and Miss Kasana Sukapirom, Office for Research and Development, Mahidol University, for kind advice and the opportunity to train to perform DNA damage analysis using a flow cytometer. This paper has no commercial interest with any companies.

References

1 Gandini L, Lombardo F, Paoli D, Caruso F, Eleuteri P, *et al*. Full-term pregnancies achieved with ICSI despite high levels of sperm chromatin damage. Hum Reprod 2004; 19: 1409–17.
2 Lopes S, Jurisicova A, Sun JG, Casper RF. Reactive oxygen species: potential cause for DNA fragmentation in human spermatozoa. Hum Reprod 1998; 13: 896–900.
3 Sailer BL, Jost LK, Evenson DP. Mammalian sperm DNA susceptibility to *in situ* denaturation associated with the presence of DNA strand breaks as measured by the terminal deoxynucleotidyl transferase assay. J Androl 1995; 16: 80–7.
4 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.
5 Gorczyca W, Traganos F, Jesionowska H, Darzynkiewicz Z. Presence of DNA strand breaks and increased sensitivity of DNA *in situ* to denaturation in abnormal human sperm cells: anology to apoptosis of somatic cells. Exp Cell Res1993; 207: 202–5.
6 Cho C, Jung-Ha H, Willis WD, Goulding EH, Stein P, *et al*. Protamine 2 deficiency leads to sperm DNA damage and embryo death in mice. Biol Reprod 2003; 69: 211–7.
7 Ahmadi A, Ng SC. Destruction of protamine in human sperm inhibits sperm binding and penetration in the zona-free hamster penetration but increases sperm head decondensation and male pronuclear formation in the hamster-ICSI assay. J Assist Reprod Genet 1999; 16: 128–32.
8 Iizuka R, Kaneko S, Kobanawa K, Kobayashi T. Washing and

concentration of human semen by Percoll density gradients and its application to AIH. Arch Androl 1988; 20: 117–24.
9 Mathieu C, Guerin JH, Gille Y, Pinatel MC, Lornage J, *et al*. Separation of spermatozoa using Percoll gradients: value for *in vitro* fertilization. J Gynecol Obstel Biol Reprod 1988; 17: 237–41.
10 Punjabi U, Gerris J, van Bijlen J, Delbeke L, Gielis M, *et al*. Comparison between different pre-treatment techniques for sperm recovery prior to intrauterine in sememination, GIFT or IVF, Hum Reprod 1990; 5: 75–83.
11 Claassens OE, Kaskar K, Coetzee K, Lombard CJ, Franken DR, *et al*. Comparison of motility characteristics and normal sperm morphology of human semen samples separated by Percoll density gradient centrifugation. Arch Androl 1996; 36: 127–32.
12 Berger T, Marrs RP, Moyer DL. Comparison of techniques for selection of motile spermatozoa. Fertil steril 1985; 43: 268–73.
13 McClure RD, Nunes L, Tom R. Semen manipulation: improved sperm recovery and function with a two-layer Percoll gradient. Fertil Steril 1989; 51: 874–7.
14 Bongso A, Jarina AK, Ho J, Ng SC, Ratnam SS. Comparative evaluation of three sperm-washing methods to improve sperm concentration and motility in frozen-thawed oligozoospermic and normozoospermic samples. Arch Androl 1993; 31: 223–30.
15 Moohan JM, Lindsay KS. Spermatozoa selected by a discontinuous Percoll density gradient exhibit better motion characteristics, more hyperactivation, and longer survival than direct swim-up. Fertil Steril 1995; 64: 160–5.
16 Simeon NM, Rives N, Masse L, Cherallier F, Mace B. Comparison of six density gradient media for selection of cryopreserved donor spermatozoa. J Androl 2004; 25: 881–4.
17 Sterckx J, Verheyen G, De Vos A, Abbeel VD, Van Steirteghem A. Comparision of two commercial density gradient on sperm motility, fertilization and embryo quality after *in vitro* fertilization on sibling oocyte. Abstracts of the 22nd Annual Meeting of the ESHRE, 2006: 126.
18 Allamaneni SS, Agarwal A, Rama S, Ranganathan P, Sharma RK. Comparative study on density gradients and swim-up preparation techniques utilizing neat and cryopreserved spermatazoa. Asian J Androl 2005; 7: 86–92.
19 World Health Organization. WHO Laboratory Manual for the Examination of Human Semen-Cervical Mucus Interaction. 4th ed. Cambridge: Cambridge University Press; 1999.
20 Kruger TF, Coetzee K. The role of sperm morphology in assisted reproduction. Hum Reprod Update 1999; 5: 172–8.
21 Fischer HC, Al-Hasani S, Rosenhaum P, Schmidt W, Hammadeh. Sperm quality before and after semen preparation with PureSperm? or Isolate? and their effect on IVF outcome. Abstracts of the 22nd Annual Meeting of the ESHRE 2006: 125–6.
22 Zhang HS, Lui SM, Ma CY, Wang L, Li X, *et al*. Early apoptotic changes in human spermatozoa and their relationships with conventional semen parameters and sperm DNA fragmentation. Asian J Androl 2008; 10: 227–35.