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GAMETE BIOLOGY

Sperm DNA and chromatin integrity in semen samples used for intrauterine insemination

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Abstract
Background Sperm DNA damage is associated with male infertility but whether normozoospermic infertile men also have DNA damage is unknown.
Objective To evaluate sperm DNA and chromatin integrity in men with mild male factor infertility.
Design, setting and participants Prospective study of 102 consecutive men (78 normozoospermic, 15 asthenozoospermic, 9 oligozoospermic) enrolled for intrauterine insemination (IUI) and 15 fertile controls.
Outcome measurements and statistical analysis Standard semen parameters and sperm chromatin and DNA integrity were assessed and compared between groups. Sperm chromatin quality was assessed by (1) aniline blue staining (AB is specific to histone lysines), (2) iodoacetamide fluorescein fluorescence (IAF targets free protamine sulfhydryl groups) and (3) sperm chromatin structure assay (SCSA) with the results expressed as % DNA fragmentation index (%DFI).
Results and limitations The mean (±SD) percentage of spermatozoa with positive IAF fluorescence was significantly higher in the IUI population compared to fertile controls (17 %±10 % vs. 8 %±6 %, $P=0.0011$) and also in the normozoospermic subset ($n=78$) compared to controls (16 %±9 % vs. 8 %±6 %, $P<0.0001$, ANOVA). We also observed a trend toward lower %progressive motility, and higher %AB staining and %DFI in the IUI group compared to controls. We observed significant relationships between sperm %DFI and progressive motility ($r=-0.40$, $P<0.0001$) and between positive AB staining and IAF fluorescence ($r=0.58$, $P<0.0001$).
Conclusions The data indicate that sperm chromatin integrity may be abnormal in men enrolled in IUI treatment cycles, despite the fact that most of these men are normozoospermic.

Keywords Sperm DNA · Intrauterine insemination · Male infertility · Normozoospermia · Semen · Sperm chromatin

Capsule In a prospective study of 102 consecutive couples presenting for IUI, we have found that sperm chromatin compaction is often abnormal in the men, many of whom are normozoospermic. The clinical impact of these findings (e.g. effect on reproductive outcomes) remains to be verified.

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Introduction

The exact causes of male infertility are poorly understood, with nearly half of all cases deemed idiopathic [1]. Over the past decade, a considerable amount of work has been directed toward understanding the role played by DNA damage and chromatin integrity in male infertility, in terms of the etiology and pathophysiology. Multiple theories have been proposed to better understand it, and the three main emerging theories are abortive apoptosis [2], defective sperm maturation [3–5], and oxidative stress [6–8]. Although various assays have been developed to assess sperm DNA damage and chromatin integrity, the ideal marker of sperm DNA damage in the evaluation of the infertile man has not been found [9, 10].

Previous studies have shown that sperm DNA damage is elevated in patients with poor semen parameters [11, 12].

Moreover, the extent of the DNA damage increases with an increasing number of semen abnormalities [11, 12]. However, no studies have specifically examined the sperm chromatin and DNA damage of men with mild male factor infertility (e.g. normozoospermic infertile men). As such, the clinical value of sperm chromatin and DNA markers in the evaluation of men with mild male factor infertility is not known.

The purpose of our study was to evaluate sperm DNA and chromatin damage in semen samples of men enrolled in IUI treatment cycles, many of which are normozoospermic. We also sought to evaluate the relationship between markers of sperm DNA and chromatin damage and semen parameters in these men.

Materials and methods

Materials

Acridine orange (AO) was purchased from PolySciences (Warrington, PA). 5-iodoacetamide-fluorescein (IAF) was purchased from Invitrogen (Burlington, ON, Canada). Unless otherwise stated, all other chemicals were obtained from Sigma Chemical Co (St. Louis, MO) and were reagent grade or higher.

Study population

This is a prospective study of 102 consecutive non-azoospermic infertile men enrolled in IUI treatment cycles at the OVO fertility clinic between January 2010 and December 2010. These men and their partners were enrolled in IUI cycles by the treating reproductive endocrinologist based on a prior clinical evaluation (including a diagnostic semen analysis) showing normozoospermia or mild oligo-asthenoteratozoospermia (mild male factor). The controls were 15 fertile, healthy volunteers selected for their excellent semen quality. All participants were informed of the study and signed a consent form, as per the ethics board guidelines.

Semen analysis

The participants provided semen samples (for their IUI treatment cycle) by masturbation after 2–5 days of sexual abstinence. As part of the OVO clinic's standard practice, a semen analysis is routinely obtained from a small aliquot of the sample used for IUI. The IUI semen analysis includes assessment of semen volume, sperm concentration and progressive motility (by computer-assisted semen analyzer) but sperm morphology is not assessed as part of this evaluation [13]. The patients were categorized based on the IUI semen analysis results (normozoospermic, asthenozoospermic and oligo- or oligoasthenozoospermic). We elected not to categorize patients based on the diagnostic semen analyses (these were conducted weeks or months before the IUI treatment) because these semen analyses were done in several different clinical laboratories each using different methods of analysis and different morphology criteria.

Prior to sperm processing for the IUI treatment, a small aliquot of raw semen (between 25 and 100 μL of semen, containing approximately 1 to 2 million spermatozoa but no more than 5 % of the entire semen sample) was collected from the original sample and frozen at −70 °C for the subsequent evaluation of sperm chromatin structure assay (SCSA) parameters and cytochemical tests of sperm chromatin integrity).

Sperm chromatin structure assay

Sperm DNA damage was assessed using the sperm chromatin structure assay (SCSA), and the results were expressed as % DNA fragmentation index – % DFI (an index of DNA damage), as described previously [14–16]. Stored semen samples were thawed on ice and treated for 30 s with 400 μL of a solution of 0.1 % Triton X-100, 0.15 m NaCl, and 0.08 N HCl (pH 1.2). After 30 s, 1.2 mL of staining buffer (6 l g/mL acridine orange (AO), 37 ml citric acid, 126 ml Na_2HPO_4, 1 ml disodium EDTA, 0.15 M NaCl (pH 6.0)) was mixed into the test tube. The sample was placed in a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA), and the flow rate was adjusted to ensure an optimal sheath/sample flow rate ratio. Measurements were taken exactly 3 min after AO staining. A minimum of 5,000 cells from 2 aliquots of each sample were analyzed by a FACS scan interfaced with a data handler (CELLQUEST 3.1; Becton Dickinson) on a Power Macintosh 7600/132 computer (Cupertino, CA, USA). WinList (Verity Software House Inc., Topsham, ME, USA) was used to generate cytogram (red vs. green fluorescence) and histogram (total cells vs. number showing DFI) plots as well as to take percentage DFI and percentage HDS readings. The mean of the two values was reported. The variability of the replicate SCSA measures (percentage DFI and percentage HDS) was <5 %. We (and others) have shown that the SCSA results are similar whether tested on fresh or previously frozen samples (frozen at −7 °C without cryoprotectant) [12, 14, 16].

Cytochemical tests of sperm chromatin: aniline blue and iodoacetamide fluorescein

Thawed semen samples were fixed with 70 % ethanol and kept at −20 °C before further processing. Smears were prepared from the fixed semen samples, left to air-dry at 20 °C for 30 min and immediately stained. For aniline blue (AB) staining [17], the smears were incubated with the dye (5 % AB in 4 % acetic acid) for 5 min, washed three times with dH2O and mounted with glycerol. For iodoacetamide-fluorescein (IAF)

fluorescence [17, 18], the smears were incubated with 0.1 M Tris (pH 6.8) for 5 min and then with 0.1 mM IAF for 15 min. The IAF-stained smears were rinsed briefly with dH2O, washed with Tris and then mounted with DABCO.

For both the AB and IAF staining, at least 200 spermatozoa were counted per slide. The AB and IAF staining evaluations were blinded and repeat measurements were obtained two separate investigators to assess inter-observer variability. The inter-observer variability for AB and IAF staining measures were both <10 %. The same grading systems as reported by de Lamirande et al. [17] were used. For AB staining test, spermatozoa were divided into three categories: intense (dark blue stain over the entire head), intermediate (dark staining in the post-acrosomal region only) and pale (very pale staining over the entire head). For the IAF test, fluorescence was graded as intense (bright fluorescence of the entire head), intermediate (bright fluorescence of the post-acrosomal region only) and pale (very pale fluorescence of the entire head). In the present study, the results of the IAF and AB tests were reported as %IAF fluorescence (reflecting the % of sperm with bright fluorescence of the entire head) and %AB staining (reflecting the % of sperm with dark blue stain over the entire head), respectively. We have compared the AB and IAF assay results in fresh and previously frozen samples (frozen at −7 °C) and have found a low coefficient of variation for both tests (7.5 % for AB and 9.2 % for IAF, respectively).

Data analysis

Results are expressed as mean ± SD. Inter-group (fertile controls and infertile men enrolled in IUI treatment) differences in sperm parameters were assessed by parametric and non-parametric tests as appropriate (one way ANOVA and Mann–Whitney rank sum test). The relationships between parameters were examined using linear regression techniques with Pearson's correlation coefficient. All hypothesis testing was two-sided with a probability value of 0.05 deemed as significant. We set the cutoff (critical) values for high %IAF and %AB staining based on the mean+2 SDs of the control population %IAF and %AB staining. The cutoff value for high %DFI (>15 %) was based on a prior publication [14]. Statistical analysis was performed using SSPS version 20 (SPSS Inc, Chicago, USA).

Results

Semen samples were collected from 102 infertile men enrolled for IUI treatment (78 normozoospermic, 15 asthenozoospermic, and 9 oligo- or oligoasthenozoospermic men based on the IUI semen analysis) and 15 fertile controls with normal sperm parameters. Of the 78 normozoospermic men, 60 were from couples with unexplained infertility and 18 from couples with mild female factor-infertility (reduced ovarian reserve or polycystic ovary syndrome). The mean (±SD) sperm concentration was significantly higher in the fertile control compared to the group IUI population (see Table 1). The mean (±SD) %IAF fluorescence was significantly higher in the global IUI population ($n=102$) compared to the fertile control group (17 %± 10 % vs. 8 %±6 %, $P=0.0011$). However, there was no statistically significant difference in %progressive motility, %AB staining and %DFI between the global IUI group ($n=102$) and the fertile controls (Table 1). Moreover, there was no statistically significant difference in %progressive motility, %AB staining, %IAF fluorescence and %DFI between the 60 normozoospermic men from couples with unexplained infertility and the 18 men from couples with mild female factor-infertility (data not shown). The prevalence of semen samples with elevated %IAF (>20 % cutoff), %DFI (>15 % cutoff) and %AB (>31 % cutoff) in the IUI population ($n=102$) was 32 %, 20 % and 7 %, respectively.

Subgroup analysis of the 102 infertile men (78 normozoospermic, 15 asthenozoospermic, and 9 oligozoospermic) demonstrated that the mean %IAF fluorescence, %AB staining and %DFI were significantly higher in the asthenozoospermic subset ($n=15$) compared to the fertile controls (see Table 2). The mean %IAF fluorescence was also significantly higher in the normozoospermic and oligozoospermic subsets compared to controls (see Table 2). The prevalence of semen samples with elevated %IAF (>20 % cutoff), %DFI (>15 % cutoff) and %AB (>31 % cutoff) in the normozoospermic subset ($n=78$) was 28 %, 12 % and 3 %, respectively.

We observed significant relationships between sperm %DFI and %progressive motility ($r=-0.40$, $P<0.0001$) and between %AB staining and %IAF fluorescence ($r=0.58$, $P<0.0001$) by univariate linear regression analysis.

Table 1 Mean (±SD) sperm %AB staining, %IAF fluorescence, %DFI, and %HDS in the IUI patients and controls

Subgroup	IUI	Ctl	p-value[a]
n	102	15	
Concentration (million/ml)	76±59	176±228	0.0011
Progressive motility (%)	55±23	64±11	>0.05
%AB staining	17±10	12±9	>0.05
%IAF fluorescence	17±10	8±6	0.0009
%DFI	10±7	6±3	>0.05
%HDS	5±3	6±3	>0.05

Values are means ± SD

AB aniline blue staining (percentage of dark staining), *IAF* iodoacetimide fluorescence (percentage of intense fluorescence), *DFI* DNA fragmentation index, *HDS* high DNA stainability

[a] Mann–Whitney rank sum test

Table 2 Mean (±SD) sperm %AB staining, %IAF fluorescence, %DFI, and %HDS in the 3 IUI patient subgroups (normozoospermic, asthenozoospermic, oligo + oligoasthenozoospermic) and controls

Subgroup	N	A	O + OA	Ctl	p-value
n	78	15	9	15	
%AB staining	15±9[a]	23±12[b]	21±15[a,b]	12±9[a]	0.0095
%IAF fluorescence	16±9[a]	22±12[b]	21±13[a]	8±6[c]	0.0009
%DFI	9±6[a]	15±7[b]	11±9[a,b]	6±3[a]	0.0017
%HDS	5±2[a]	6±4[a]	6±3[a]	6±3[a]	>0.05

Values are means ± SD

N normozoospermia, *A* asthenozoospermia, *O + OA* oligozoospermia and oligoasthenozoospermia, *Ctls* fertile controls, *AB* aniline blue staining (percentage of dark staining), *IAF* iodoacetimide fluorescence (percentage of intense fluorescence), *DFI* DNA fragmentation index, *HDS* high DNA stainability

[a,b] Different letters indicate significant difference between subgroups (Kruskal-Wallis one-way ANOVA on ranks)

Discussion

Men enrolled in IUI treatment cycles typically have normal or very mildly reduced sperm concentration and/or motility at the time of the IUI cycle. Nonetheless, we have observed that a significant subset of these men (more than 30 % of the men) have detectable defects in sperm chromatin integrity and that the mean %IAF fluorescence was significantly higher in the men enrolled for IUI treatment compared to the fertile controls. As would be expected from previous observations, we have shown that the degree of sperm DNA and chromatin defects was more pronounced in the subset of men with asthenozoospermia when compared to normozoospermic men [11]. This is in keeping with reports showing that patients with even a single abnormal sperm parameter have a higher %DFI and poorer chromatin compaction than fertile controls [19, 20]. Moreover, in our population of men with mild sperm defects, sperm %DFI was inversely related to % progressive motility. These data are in line with several reports showing a significant inverse relationship between sperm DNA damage and progressive motility, although, prior studies evaluated unselected infertile men, many of whom have significantly poorer semen parameters than the men in this study [11, 21–24].

The current study highlights the fact that infertile men with normal semen parameters may have abnormal sperm chromatin. Indeed, the mean %IAF staining was significantly higher in samples from normozoospermic men (most of whom have unexplained infertility) than samples from fertile controls and, moreover, close to 30 % of samples from normozoospermic men had an abnormal sperm chromatin or DNA test result (above the cutoff value or 2 SDs above the control population mean). These data suggest that sperm chromatin testing may help uncover a sperm defect in infertile men with normal semen parameters. One must note that our data are weakened by the fact that sperm morphology was not formally assessed as part of the IUI semen analysis although most of these men would have had a normal morphology on the diagnostic semen analysis (done several weeks prior to the IUI cycle). However, it is not known whether a mild but detectable sperm chromatin defect in a man with normal semen parameters will have an impact on reproductive outcomes.

We have previously shown that both IAF and AB staining can detect defects in sperm chromatin compaction and, indirectly, assess sperm nuclear maturity [17]. These two cytochemical tests (IAF fluorescence and AB staining) are believed to assess (1) the relative accessibility of the dye to the nuclear target (AB preferentially binds to histone lysines and IAF preferentially binds to free protamine sulfhydryl [SH] groups) and, to some extent, (2) the relative levels of the nuclear target (histone content and free SH levels) [17]. Therefore, as expected, we have found a significant correlation between %AB staining and %IAF fluorescence ($r=0.58$) despite the fact that AB and IAF have vastly different chromatin targets. As such, our observations further support the premise that chromatin compaction and accessibility to the nuclear target is a common and important element of cytochemical chromatin integrity tests such as IAF and AB.

We have observed a wide variability in the test results of the different sperm DNA and chromatin assays in our population of men with mild male-factor infertility (the prevalence of semen samples with elevated %IAF, %DFI and %AB was 32 %, 20 % and 7 %, respectively). This variability may be due to the fact that the etiology of sperm chromatin and DNA damage is multifactorial (aberrant spermatogenesis, abortive apoptosis, oxidative stress) and, therefore, the variability in the test results of the different assays may simply reflect this [25–27]. In our study, IAF fluorescence was a better discriminator of male fertility potential than the other two markers of sperm chromatin integrity (mean %IAF staining was significantly higher in the samples from normozoospermic men compared to the samples from fertile controls). A possible explanation is that a mild defect in sperm chromatin compaction may be more readily detectable by IAF fluorescence than by AB staining (two comparable cytochemical assays) because IAF is a smaller molecule (MW~515) than AB (MW~803). Smaller molecules will typically penetrate or stain more readily than larger molecules [28]. The difference may also be related to the nature of the assays (IAF mainly targets free SH groups and AB mainly targets histone lysines). These observations should prompt larger studies of men with different etiologies of infertility to further assess the potential clinical value of the various sperm DNA and chromatin assays and to specifically determine whether the greater sensitivity of IAF fluorescence (observed in this study) is clinically relevant. Pregnancy outcome data will

help better define the relative clinical value of the three different sperm DNA and chromatin assays used in this study.

Sperm chromatin and/or DNA damage has been associated with reduced male reproductive potential and may be a crude indicator of genetic mutations arising during spermiogenesis [29, 30]. Although the ASRM Practice Committee [31] does not recommend routine use of sperm DNA tests, several studies have demonstrated that sperm chromatin and/or DNA damage is associated with a very low potential for natural fertility and a prolonged time to pregnancy [14, 32, 33]. Sperm DNA damage has been shown to adversely affect intra-uterine insemination outcomes and to a lesser degree IVF pregnancy rates, but not IVF/ICSI pregnancy rates [14, 31–37]. Sperm DNA damage has also been associated with a significant increase in the rate of pregnancy loss after IVF and ICSI [38, 39]. To date, little is known regarding the influence of sperm DNA damage on post-natal health because of the paucity of clinical (human) studies on this subject [40]. In experimental studies in mice, sperm DNA damage has been associated with chromosomal abnormalities, developmental loss, reduced longevity and birth defects [41]. In humans, advanced paternal age is associated with sperm chromatin damage and an increased risk of de novo gene mutations in sperm but whether these de novo mutations in sperm are related to the global integrity of the sperm chromatin, as measured by tests such as the sperm chromatin structure assay, remains to be verified [29, 30, 42].

Conclusion

We have shown that sperm chromatin compaction is frequently abnormal in men enrolled in IUI, many of whom are normozoospermic. However, the clinical value of these findings would be strengthened by large, prospective studies with reproductive outcomes.

References

1. Brandes M, Hamilton CJ, de Bruin JP, Nelen WL, Kremer JA. The relative contribution of IVF to the total ongoing pregnancy rate in a subfertile cohort. Hum Reprod. 2010;25(1):118–26.
2. Sakkas D, Mariethoz E, Manicardi G, Bizzaro D, Bianchi PG, Bianchi U. Origin of DNA damage in ejaculated human spermatozoa. Rev Reprod. 1999;4(1):31–7.
3. Marcon L, Boissonneault G. Transient DNA strand breaks during mouse and human spermiogenesis new insights in stage specificity and link to chromatin remodeling. Biol Reprod. 2004;70(4):910–8.
4. McPherson S, Longo FJ. Chromatin structure-function alterations during mammalian spermatogenesis: DNA nicking and repair in elongating spermatids. Eur J Histochem. 1993;37(2):109–28.
5. Sakkas D, Manicardi G, Bianchi PG, Bizzaro D, Bianchi U. Relationship between the presence of endogenous nicks and sperm chromatin packaging in maturing and fertilizing mouse spermatozoa. Biol Reprod. 1995;52(5):1149–55.
6. Aitken J, Krausz C, Buckingham D. Relationships between biochemical markers for residual sperm cytoplasm, reactive oxygen species generation, and the presence of leukocytes and precursor germ cells in human sperm suspensions. Mol Reprod Dev. 1994;39(3):268–79.
7. Greco E, Scarselli F, Iacobelli M, Rienzi L, Ubaldi F, Ferrero S, et al. Efficient treatment of infertility due to sperm DNA damage by ICSI with testicular spermatozoa. Hum Reprod. 2005;20(1):226–30.
8. Moskovtsev SI, Jarvi K, Mullen JB, Cadesky KI, Hannam T, Lo KC. Testicular spermatozoa have statistically significantly lower DNA damage compared with ejaculated spermatozoa in patients with unsuccessful oral antioxidant treatment. Fertil Steril. 2010;93(4):1142–6.
9. Barratt CL, Aitken RJ, Bjorndahl L, Carrell DT, de Boer P, Kvist U, et al. Sperm DNA: organization, protection and vulnerability: from basic science to clinical applications—a position report. Hum Reprod. 2010;25(4):824–38.
10. Chohan KR, Griffin JT, Lafromboise M, De Jonge CJ, Carrell DT. Comparison of chromatin assays for DNA fragmentation evaluation in human sperm. J Androl. 2006;27(1):53–9.
11. Moskovtsev SI, Willis J, White J, Mullen JB. Sperm DNA damage: correlation to severity of semen abnormalities. Urology. 2009;74(4):789–93.
12. Zini A, Fischer MA, Sharir S, Shayegan B, Phang D, Jarvi K. Prevalence of abnormal sperm DNA denaturation in fertile and infertile men. Urology. 2002;60(6):1069–72.
13. WHO. WHO laboratory manual for the examination of human semen and sperm-cervical mucus interaction. 4th ed. Cambridge: UCUP; 1999.
14. Evenson DP, Jost LK, Marshall D, Zinaman MJ, Clegg E, Purvis K, et al. Utility of the sperm chromatin structure assay as a diagnostic and prognostic tool in the human fertility clinic. Hum Reprod. 1999;14(4):1039–49.
15. Zini A, Azhar R, Baazeem A, Gabriel MS. Effect of microsurgical varicocelectomy on human sperm chromatin and DNA integrity: a prospective trial. Int J Androl. 2011;34(1):14–9.
16. Zini A, Bielecki R, Phang D, Zenzes MT. Correlations between two markers of sperm DNA integrity, DNA denaturation and DNA fragmentation, in fertile and infertile men. Fertil Steril. 2001;75(4):674–7.
17. de Lamirande E, San Gabriel MC, Zini A. Human sperm chromatin undergoes physiological remodeling during in vitro capacitation and acrosome reaction. J Androl. 2012;33(5):1025–35.
18. Chatterjee S, de Lamirande E, Gagnon C. Cryopreservation alters membrane sulfhydryl status of bull spermatozoa: protection by oxidized glutathione. Mol Reprod Dev. 2001;60(4):498–506.
19. Saleh RA, Agarwal A, Sharma RK, Said TM, Sikka SC, Thomas Jr AJ. Evaluation of nuclear DNA damage in spermatozoa from infertile men with varicocele. Fertil Steril. 2003;80(6):1431–6.
20. Saleh RA, Agarwal A, Nada EA, El-Tonsy MH, Sharma RK, Meyer A, et al. Negative effects of increased sperm DNA damage in relation to seminal oxidative stress in men with idiopathic and male factor infertility. Fertil Steril. 2003;79 Suppl 3:1597–605.
21. Chi HJ, Chung DY, Choi SY, Kim JH, Kim GY, Lee JS, et al. Integrity of human sperm DNA assessed by the neutral comet assay and its relationship to semen parameters and clinical outcomes for the IVF-ET program. Clin Exp Reprod Med. 2011;38(1):10–7.
22. Oliveira JB, Massaro FC, Baruffi RL, Mauri AL, Petersen CG, Silva LF, et al. Correlation between semen analysis by motile sperm organelle morphology examination and sperm DNA damage. Fertil Steril. 2010;94(5):1937–40.
23. Varghese AC, Bragais FM, Mukhopadhyay D, Kundu S, Pal M, Bhattacharyya AK, et al. Human sperm DNA integrity in normal and abnormal semen samples and its correlation with sperm characteristics. Andrologia. 2009;41(4):207–15.

24. Cohen-Bacrie P, Belloc S, Menezo YJ, Clement P, Hamidi J, Benkhalifa M. Correlation between DNA damage and sperm parameters: a prospective study of 1,633 patients. Fertil Steril. 2009;91(5):1801–5.
25. De Iuliis GN, Thomson LK, Mitchell LA, Finnie JM, Koppers AJ, Hedges A, et al. DNA damage in human spermatozoa is highly correlated with the efficiency of chromatin remodeling and the formation of 8-hydroxy-2′-deoxyguanosine, a marker of oxidative stress. Biol Reprod. 2009;81(3):517–24.
26. Leduc F, Nkoma GB, Boissonneault G. Spermiogenesis and DNA repair: a possible etiology of human infertility and genetic disorders. Syst Biol Reprod Med. 2008;54(1):3–10.
27. Sakkas D, Seli E, Bizzaro D, Tarozzi N, Manicardi GC. Abnormal spermatozoa in the ejaculate: abortive apoptosis and faulty nuclear remodelling during spermatogenesis. Reprod Biomed Online. 2003;7(4):428–32.
28. Darzynkiewicz Z. Critical aspects in analysis of cellular DNA content. Curr Protoc Cytom. 2011;Chapter 7:Unit 7 2.
29. Gregoire MC, Massonneau J, Simard O, Gouraud A, Brazeau MA, Arguin M, et al. Male-driven de novo mutations in haploid germ cells. Mol Hum Reprod. 2013;19(8):495–9.
30. Kong A, Frigge ML, Masson G, Besenbacher S, Sulem P, Magnusson G, et al. Rate of de novo mutations and the importance of father's age to disease risk. Nature. 2012;488(7412):471–5.
31. The clinical utility of sperm DNA integrity testing: a guideline. Fertil Steril. 2013;99:673–7.
32. Giwercman A, Lindstedt L, Larsson M, Bungum M, Spano M, Levine RJ, et al. Sperm chromatin structure assay as an independent predictor of fertility in vivo: a case–control study. Int J Androl. 2010;33:e221–7.
33. Spano M, Bonde JP, Hjollund HI, Kolstad HA, Cordelli E, Leter G. Sperm chromatin damage impairs human fertility. The Danish First Pregnancy Planner Study Team. Fertil Steril. 2000;73(1):43–50.
34. Bungum M, Humaidan P, Axmon A, Spano M, Bungum L, Erenpreiss J, et al. Sperm DNA integrity assessment in prediction of assisted reproduction technology outcome. Hum Reprod. 2007;22(1):174–9.
35. Collins JA, Barnhart KT, Schlegel PN. Do sperm DNA integrity tests predict pregnancy with in vitro fertilization? Fertil Steril. 2008;89(4):823–31.
36. Zini A. Are sperm chromatin and DNA defects relevant in the clinic? Syst Biol Reprod Med. 2011;57(1–2):78–85.
37. Zini A, Sigman M. Are tests of sperm DNA damage clinically useful? Pros and cons. J Androl. 2009;30(3):219–29.
38. Robinson L, Gallos ID, Conner SJ, Rajkhowa M, Miller D, Lewis S, et al. The effect of sperm DNA fragmentation on miscarriage rates: a systematic review and meta-analysis. Hum Reprod. 2012;27(10):2908–17.
39. Zini A, Boman JM, Belzile E, Ciampi A. Sperm DNA damage is associated with an increased risk of pregnancy loss after IVF and ICSI: systematic review and meta-analysis. Hum Reprod. 2008;23(12):2663–8.
40. Bungum M, Bungum L, Lynch KF, Wedlund L, Humaidan P, Giwercman A. Spermatozoa DNA damage measured by sperm chromatin structure assay (SCSA) and birth characteristics in children conceived by IVF and ICSI. Int J Androl. 2012;35(4):485–90.
41. Fernandez-Gonzalez R, Moreira PN, Perez-Crespo M, Sanchez-Martin M, Ramirez MA, Pericuesta E, et al. Long-term effects of mouse intracytoplasmic sperm injection with DNA-fragmented sperm on health and behavior of adult offspring. Biol Reprod. 2008;78(4):761–72.
42. Wyrobek AJ, Eskenazi B, Young S, Arnheim N, Tiemann-Boege I, Jabs EW, et al. Advancing age has differential effects on DNA damage, chromatin integrity, gene mutations, and aneuploidies in sperm. Proc Natl Acad Sci U S A. 2006;103(25):9601–6.