Journal of Assisted Reproduction and Genetics, Vol. 21, No. 4, April 2004 (© 2004)

Physiology

Human Cervical Mucus Can Act *in Vitro* as a Selective Barrier Against Spermatozoa Carrying Fragmented DNA and Chromatin Structural Abnormalities

P.G. Bianchi,[1] A. De Agostini,[1] J. Fournier,[1] C. Guidetti,[2] N. Tarozzi,[2] D. Bizzaro,[3] and G.C. Manicardi[2,4]

Submitted September 8, 2003; accepted February 27, 2004

***Purpose*:** We have carried out experiments to determine if human cervical mucus can act as an *in vitro* selective barrier against spermatozoa morphologically normal that carry genetic structural abnormalities.
***Methods*:** Sperm chromatin abnormalities have been evaluated by Chromomycin A_3 and "endogenous" nick translation.
***Results*:** The data obtained have shown that spermatozoa possessing higher levels of DNA protamination are more proficient in crossing the cervical mucus barrier. Moreover, the levels of positivity to endogenous nick translation treatment was practically zero in such spermatozoa.
***Conclusions*:** We suggest that sperm penetration of cervical mucus could be used to select sperm preparations free of fragmented DNA or chromatin structural abnormalities for assisted reproduction.

KEY WORDS: Chromatin abnormalities; Chromomycin A_3; human cervical mucus; *in situ* nick translation; spermatozoa.

INTRODUCTION

The passage of spermatozoa across human cervical mucus *in vitro* has been used to provide important predicting information regarding sperm function (1–3). An important clinical use of cervical mucus is in the diagnosis of sperm autoimmunity since spermatozoa heavily coated with antisperm antibody are unable to penetrate it (4). Traditional semen parameters such as concentration, motility, and morphology of spermatozoa have also been demonstrated to influence sperm-cervical mucus penetration (2,5–11).

During the last decade, a number of studies have shown that variable but consistent fractions of human spermatozoa not always detectable on the basis of classic morphological parameters, contain serious chromatin structural abnormalities and DNA strand breaks (12–18). The existence of an association between sperm chromatin malformation and male infertility has been repeatedly observed in humans and structural chromatin abnormalities have been found to exert negative effects both on the percentage of fertilization and on embryonic development (17,19–21).

This study aims to determine the potential of human cervical mucus as an *in vitro* selective barrier for spermatozoa carrying genetic and structural abnormalities, respectively evaluated by "endogenous" nick translation (NT) and Chromomycin A_3 (CMA_3).

NT is a technique used to evaluate the presence of endogenous nicks in the DNA. Indeed, in the

[1] Clinic of Sterility, Department of Obstetrics and Gynaecology, University Hospital of Geneva, Geneva, Switzerland.
[2] Dipartimento di Scienze Agrarie, Università di Modena e Reggio Emilia, Reggio Emilia, Italy.
[3] Istituto di Biologia e Genetica, Università Politecnica delle Marche, Ancona, Italy.
[4] To whom correspondence should be addressed at Università di Modena e Reggio Emilia, Via Kennedy 17, 42100 Reggio Emilia, Italy, e-mail: manicardi.giancarlo@unimore.it.

1058-0468/04/0400-0097/0 © 2004 Plenum Publishing Corporation

presence of preexisting endogenous nicks, the DNA polymerase I can catalyze movement of the nicks along the double helix by virtue of its 5′-3′ exonucleotic activity (16). Therefore *in situ* NT can be useful to point out nuclear DNA damage.

CMA_3 is a useful tool for rapid screening of subfertility in man, as it seems to allow an indirect visualization of protamine-deficient DNA (14,15,22,23). This molecule has been shown to bind as a Mg^{2+}-coordinated dimer at the minor groove of GC-rich DNAs and to induce a conformational perturbation in the double helix resulting in a wider and shallower minor groove at its binding site (24,25). In addition, when CMA_3 binds the DNA, it also compresses its wide major groove (26), usually occupied by protamines. For these reasons the conformational arrangement adopted by the DNA-protamine complex can limit the access of CMA_3 to the minor groove. So CMA_3 can also be used as an indicator of underprotaminated spermatozoa (23).

MATERIALS AND METHODS

Preparation of Semen Samples and Slides

Semen samples were obtained from 20 men attending the Clinic of Sterility, University Hospital of Geneva, Geneva, Switzerland. All samples were randomly obtained on the basis of availability and consent. No data on specimen were available to the researchers when the experiments were performed.

Sperm parameters varied considerably and ranged from a concentration of 1×10^6 spermatozoa per mL and 10% progressive motility to 180×10^6 spermatozoa per mL and 58% progressive motility. Ejaculates from each donor were divided in two fractions. The first fraction was washed twice with PBS (137-mM NaCl, 2.7-mM KCl, 10-mM Na_2HPO_4/KH_2PO_4) Ca^{++} Mg^{++} free and centrifuged. Spermatozoa were then fixed in methyl alcohol-acetic acid 3:1, at 4°C, for 15 min and then spread on slides.

Human cervical mucus was obtained, after informed consent from healthy, fertile women undergoing ovarian stimulation for IVF or ICSI procedures, on the day of induction of ovulation just before administration of 10,000 IU of human chrorionic gonadotrophin (HCG). Only mucus of good quality was used (WHO score >12). Interaction between sperm samples and cervical mucus was random. No analysis of the selecting power of the cervical mucus of each single patient on the sperm of her partner was performed for this study.

The mucus was aspirated into a capillary tube (Aspiglaire, Biomerieux, France), one end of which was put in contact with the surface of the semen. After 3 h and 30 min of incubation at 37°C in 180 μL of T6 medium, spermatozoa able to reach the other end of the capillary tube were centrifuged to eliminate the mucus and smeared on slides. Smears were fixed in methyl alcohol-acetic acid 3:1 at 4°C, for 15 min.

The slides were treated with CMA_3 and *in situ* nick translation.

In Situ NT Assay and CMA_3 Staining

In situ NT was performed as previously described (15) by omitting the endonuclease treatments, since, as already referred, in the presence of preexisting DNA endogenous nicks, the DNA polymerase I, by virtue of its 5′-3′ exonucleolitic activity, can catalyze movement of the nicks along the double helix. The only difference from the previously described method was that Digoxigenin-11-dUTPs (Boehringer Mannheim, Switzerland) were used.

For CMA_3 staining, slides were treated for 20 min with 100 μL of CMA_3 solution (0.25 mg/mL McIlvaine buffer, pH 7.0, containing 10-mM $MgCl_2$) (15) and counterstained with DAPI. They were air-dried and mounted with a 1:1 mixture of PBS and glycerol. In nearly all cases an operator, working in the blind, examined at least 500 sperm cells on each coded slide.

Fluorescence analyses were performed using a Zeiss Axioplan Microscope (Zeiss, Germany). The nick translation and CMA_3 staining levels were predominantly of all-or-nothing type under fluorescent microscope observation carried out at 400x magnification; the rare cells showing ambiguous fluorescence were not taken into account.

RESULTS

All subjects analyzed showed variable percentages of sperm with chromatin abnormalities, detected both by CMA_3 staining and *in situ* NT.

Spermatozoa showing CMA_3 and/or *in situ* NT positivity were present both in unselected and selected sperm fractions, but all subjects analyzed showed percentages of CMA_3 positivity consistently higher than those observed after NT in both sperm fractions (see Figs. 1 and 2). Moreover, the percentages of NT positivity in selected spermatozoa were consistently lower than those present in unselected spermatozoa in all specimens (Fig. 3). The same result, with only

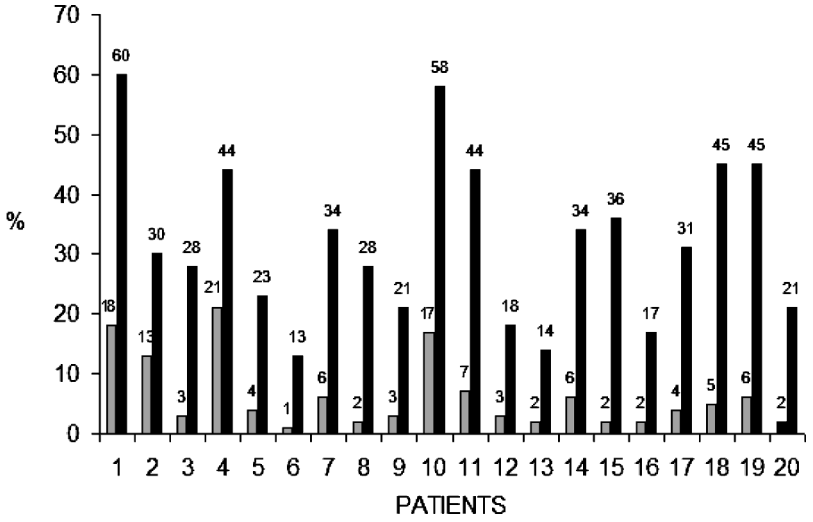

Fig. 1. Comparison between percentages of posititvity to *in situ* NT (grey) and CMA_3 (black) estimated in row spermatozoa. Statistical analysis shows that these two parameters are highly correlated: $r = 0.7471$, $P < 0.001$.

one exception, was observed after CMA_3 treatment (Fig. 4).

Statistical analysis carried out with *t* Student (pair data) showed significant differences between the unselected spermatozoa and the spermatozoa selected by cervical mucus, both with CMA_3 ($t = 5.256$; $P < 0.001$) and *in situ* NT ($t = 4.585$; $P < 0.001$). Moreover statistical analysis showed the existence of a highly significant correlation between *in situ* NT and CMA_3 positivity in unselected sperm ($r = 0.7471$, $P < 0.001$) (Fig. 5(a)), but not in spermatozoa selected by cervical mucus ($r = 0.08817$, $P < 0.7117$) (Fig. 5(b)).

DISCUSSION

In human reproduction the importance of cervical mucus is well known: alterations in mucus

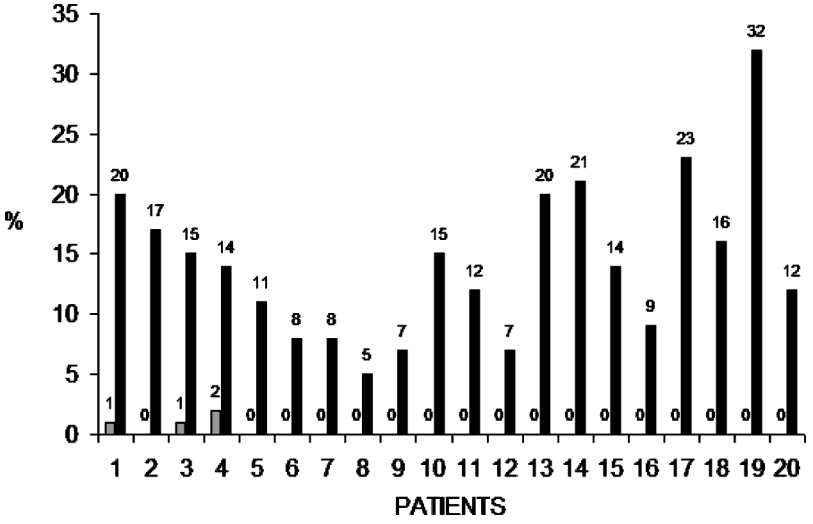

Fig. 2. Comparison between percentages of positivity to *in situ* NT (grey) and CMA_3 (black) estimated in spermatozoa selected by cervical mucus. Statistical analysis shows that there is no correlation between these two parameters: $r = 0.08817$, $p = 0.7117$.

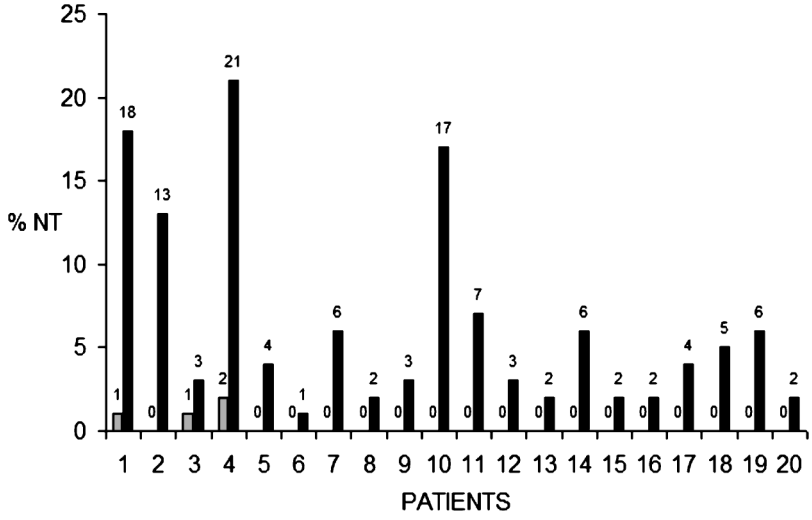

Fig. 3. Percentage of positivity to *in situ* NT in unselected spermatozoa (black) and in spermatozoa selected by cervical mucus (grey). t-test $t = 4.485$; $p < 0.01$.

biochemical properties are responsible for changes in its receptivity toward spermatozoa (27–30). Eggert-Kruse and coworkers (9) have evaluated how, in nature, the barrier represented by cervical mucus could play a selective role against spermatozoa of "bad quality." These studies showed the existence of a strong relationship between morphological abnormalities of the sperm heads and inadequate mucus sperm penetration. This is in agreement with previous experiments performed by analyzing standard sperm parameters (31,32). However the results showed that sperm morphology is only one element involved in the sperm–mucus interaction and that this process can be regulated by many other parameters.

The aim of our experiments was to evaluate the cervical mucus efficacy as a selective barrier for spermatozoa carrying chromatin structural abnormalities, without taking into consideration any of the traditional parameters so far evaluated. such as morphology, concentration, pH, and motility.

In this connection, it must be remembered that several studies have demonstrated that a variable but consistent fraction of human spermatozoa exhibits abnormalities in chromatin structure, not always

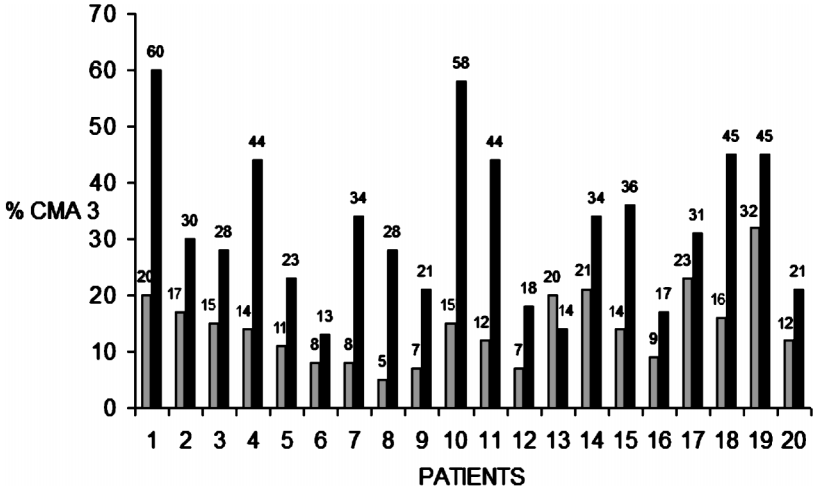

Fig. 4. Percentage of positivity to CMA_3 in unselected spermatozoa (black) and in spermatozoa selected by cervical mucus (grey). t-test $t = 5.256$; $p < 0.01$.

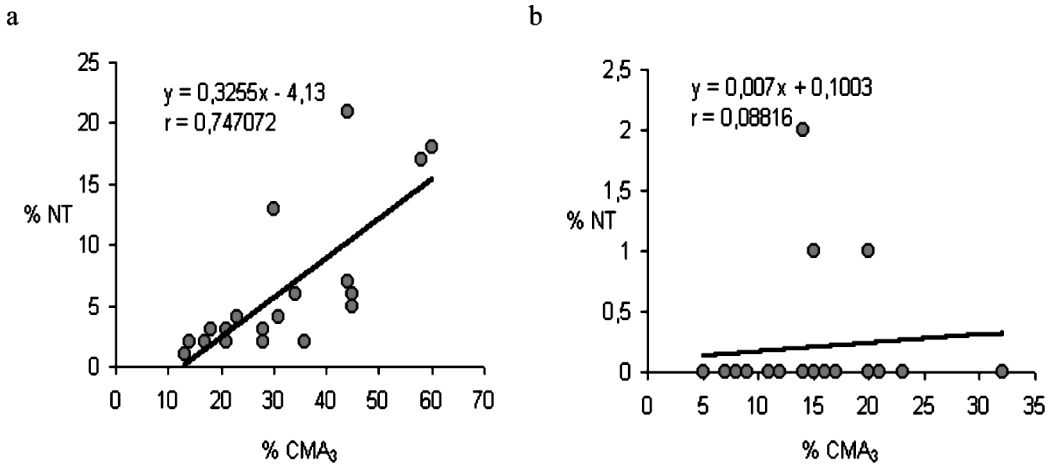

Fig. 5. Correlation between percentages of positivity to *in situ* NT and CMA_3 in unselected (a) and selected (b) spermatozoa.

detected using classical parameters of evaluation (12–18,33–35). Moreover the existence of a close relationship between chromatin and DNA structural abnormalities and male infertility has been well pointed out. Abnormalities of sperm chromatin structure have negative effects on fertilization rate, embryo development, and pregnancy outcome (17,19–21).

Our experiments have clearly shown that in vitro use of cervical mucus as a selective medium for ejaculated spermatozoa results in a definite and very significant improvement of sperm quality. Indeed, our data have demonstrated that spermatozoa showing low levels of CMA3 positivity and thus possessing high level of DNA protamination were able to cross successfully the barrier of cervical mucus. Moreover the levels of NT positivity in selected spermatozoa are near to zero indicating the occurrence of undetectable levels of DNA breaks in the DNA of such spermatozoa. On this experimental basis we suggest that cervical mucus should be used as an in vitro selection system for sperm preparation to be used in assisted reproduction techniques, because this method practically eliminates the sperm with nuclear DNA damage.

REFERENCES

1. Barrat CLR, Osborn JC, Harrison PE, Monks N, Dumphy BC, Lenton EA, Cooke ID: The hypo-osmotic swelling test and sperm mucus penetration test in determining fertilization of the human oocyte. Hum Reprod 1989;4:430–434
2. Eggert-Kruse W, Schwarz H, Rohr G, Demirakca T, Tilgen W, Runnebaum B: Sperm morphology assessment using strict criteria and male fertility under in-vivo conditions of conception. Hum Reprod 1996;11:139–146
3. Abu-Heija AT, Fleming R, Jamieson ME, Yates RW, Coutts JR: The predictive value of the sperm-cervical mucus interaction test on the outcome of in vitro fertilization and ovulation induction combined with intrauterine insemination. Aust NZJ Obstet Gynaecol 1997;37:232–234
4. Baker HW, Clarke GN, Hudson B, McBain J-C, McGowan MP, Pepperell RJ: Treatment of sperm autoimmunity in men. Clin Reprod Fertil 1983;2:55–71
5. Katz DF, Overstreet JW, Hanson FW: A new quantitative test for sperm penetration into cervical mucus. Fertil Steril 1980;33:179–186
6. Katz DF, Morales P, Samuels SJ, Overstreet JW: Mechanisms of filtration of morphologically abnormal human sperm by cervical mucus. Fertil Steril 1990;54:513–516
7. Katz DF: Human cervical mucus: Research update. Am J Obstet Gynecol 1991;165:1984–1986
8. Aitken RJ, Sutton M, Warner P, Richardson DW: Relationship between the movement characteristics of human spermatozoa and their ability to penetrate cervical mucus and zona-free hamster oocytes. J Reprod Fertil 1985;73:441–449
9. Eggert-Kruse W, Reimann-Andersen J, Rohr G, Pohl S, Tilgen W, Runnebaum B: Clinical relevance of sperm morphology assessment using strict criteria and relationship with sperm-mucus interaction *in vivo* and *in vitro*. Fertil Steril 1995;63:612–624
10. Clarke GN: A simplified quantitative cervical mucus penetration test. Hum Reprod 1997;12:1184–1187
11. Clarke GN, Garrett C, Baker HW: Quantitative sperm mucus penetration: Modified formulae for calculating penetration efficiency. Hum Reprod 1998;13:1255–1259
12. Evenson DP, Darzynkiewicz Z, Melamed MR: Relation of mammalian sperm chromatin heterogeneity to fertility. Science 1980;210:1131–1133
13. Evenson DP, Jost LK, Marshall D, Zinaman MJ, Clegg E, Purvis K, de Angelis P, Claussen OP: Utility of the sperm chromatin structure assay as a diagnostic and prognostic tool in the human fertility clinic. Hum Reprod 1999;3:1039–1049

14. Bianchi PG, Manicardi GC, Bizzaro D, Bianchi U, Sakkas D: Effect of deoxyribonucleic acid protamination on fluorochrome staining and *in situ* nick translation of murine and human nature spermatozoa. Biol Reprod 1993;49:1083–1088
15. Manicardi GC, Bianchi PG, Pantano S, Azzoni P, Bizzaro D, Bianchi U, Sakkas D: Presence of endogenous nicks in DNA of ejaculated human spermatozoa and its relationship to Chromomycin A_3 accessibility. Biol Reprod 1995;52:864–867
16. Manicardi GC, Tombacco A, Bizzaro D, Bianchi U, Bianchi PG, Sakkas D: DNA strand breaks in ejaculated human spermatozoa: Comparison of susceptibility to the nick translation and terminal transferase assays. Histochem J 1998;30:33–39
17. Sakkas D, Urner F, Bianchi PG, Bizzaro D, Wagner I, Jaquenoud N, Manicardi GC, Campana A: Sperm chromatin abnormalities can influence decondensation after intracytoplasmic sperm injection. Hum Reprod 1996;11:837–843
18. Sakkas D, Urner F, Bizzaro D, Bianchi PG, Manicardi GC, Shoukir Y, Campana A: Sperm nuclear DNA damage and altered chromatin structure: Effect on fertilization and embryo development. Hum Reprod 1998;4(Suppl):11–19
19. Qiu J, Hales BF, Robaire B: Damage to rat spermatozoal DNA after chronic cyclophosphamide exposure. Biol Reprod 1995;53:1465–1473
20. Qiu J, Hales BF, Robaire B: Effects of chronic low-dose cyclophosphamide exposure on the nuclei of rat spermatozoa. Biol Reprod 1995;52:33–40
21. Lopes S, Sun JG, Juriscova A, Meriano J, Casper RF: Sperm deoxyribonucleic acid fragmentation is increased in poor quality sperm samples and correlated with failed fertilization in intracytoplasmic sperm injection. Fertil Steril 1998;69:528–532
22. Bianchi PG, Manicardi GC, Bizzaro D, Campana A, Bianchi U, Sakkas D: Use of the guanine-cytosine (GC) specific fluorochrome Chromomycin A_3, as an indicator of poor sperm morphology. J Assist Reprod Genet 1996;13:246–250
23. Bizzaro D, Manicardi GC, Bianchi PG, Bianchi U, Mariethoz E, Sakkas D: *In situ* competition between protamine and fluorochromes for sperm DNA. Mol Hum Reprod 1998;4:127–132
24. Gao X, Patel DJ: Chromomycin dimer-DNA oligomer complex. Sequence selectivity and divalent cation specificity. Biochemistry 1990;29:10940–10956
25. Gao X, Mirau P, Patel DJ: Structure refinement of the chromomycin dimer-DNA oligomer complex in solution. J Mol Biol 1992;223:259–279
26. Goodsell DS, Kopka ML, Cascio D, Dickerson RE: Crystal structure of CATGGCCATG and its implication for A-tract bending models. Proc Natl Acad Sci USA 1993;90:2930–2934
27. Moghissi KS, Lyner FN: Studies in the human cervical mucus: Mucoids and their relation to sperm penetration. Fertil Steril 1970;21:234–239
28. Barros C, Vigil P, Herrera E, Guadarrama A, Bustos-Obregon E: *In vitro* interaction between human spermatozoa and human cervical mucus. Micr Elect Biol Cell 1983;7:13–19
29. Vigil P, Valdez E: Bases cientìficas de la planificaciòn natural de la familia. Actual Ginec Obstet 1989;3:59–67
30. Vigil P, Perez A, Neira J, Morales P: Post-partum cervical mucus: Biological and rheological properties. Hum Reprod 1991;6:475–479
31. Fredricsson B, Björk G: Morphology of postcoital spermatozoa in the cervical secretion and its clinical significance. Fertil Steril 1977;28:841–845
32. Hanson FW, Overstreet JW: The interaction of human spermatozoa with cervical mucus *in vivo*. Am J Obstet Gynaecol 1981;140:173–178
33. Foresta C, Zorzi M, Rossato M, Varotto A: Sperm nuclear instability and staining with aniline blue: Abnormal persistence of histones in spermatozoa in infertile men. Int J Androl 1992;15:330–337
34. Bianchi PG, Manicardi GC, Urner F, Campana A, Sakkas D: Chromatin packaging and morphology in ejaculated human spermatozoa: Evidence of hidden anomalies in normal spermatozoa. Mol Hum Reprod 1996;2:139–144
35. Hughes CM, Lewis SEM, McClavey-Martin VJ, Tomphson W: The effect of antioxidant supplementation during Percol preparation on human sperm DNA integrity. Mol Hum Report 1998;13:1240–1247.