ARCHIVES OF ANDROLOGY 46:99–104 (2001)

0148-5016/01 $12.00 + .00

# PREDICTIVE VALUE OF SPERM CHROMATIN CONDENSATION (ANILINE BLUE STAINING) IN THE ASSESSMENT OF MALE FERTILITY

M. E. HAMMADEH
T. ZEGINIADOV
P. ROSENBAUM

Fertility and Endocrinology Unit, Department of Obstetrics and Gynaecology, University of Saarland, Homburg/Saar, Germany

T. GEORG

Institute of Medical Biometrics and Medical Informatics, University of Saarland, Homburg/Saar, Germany

W. SCHMIDT
E. STREHLER

Fertility and Endocrinology Unit, Department of Obstetrics and Gynaecology, University of Saarland, Homburg/Saar, Germany

A case control study was carried out to determine the value of sperm chromatin condensation in the assessment of male fertility. A total of 165 semen samples from 90 patients (cases) and 75 healthy donors (control) were examined for chromatin condensation (aniline blue staining), as well as conventional sperm parameters, notably sperm morphology, sperm count, and progressive motility. Whereas only 55 ± 12.0% of the samples from the infertile patients were unstained by aniline blue (chromatin condensed), 78 ± 19.0% of the samples in the control group did not take up the stain (chromatin condensed). A significant difference ($p < .001$) was observed between the two groups. Similarly, the difference between the mean percentage of morphologically normal spermatozoa for the infertile patients (12.1 ± 1.2%) and the control (23.9 ± 1.9%) was very significant ($p < .001$). In addition, only 50 out of the 90 patients (55.5%) had a normal sperm count, whereas all the 75 (100%) were normal in the control group. By comparing between the two groups a significant difference ($p < .001$) was also observed. Furthermore, a significant difference ($p < .001$) was also found between the cases and the control with regard to the percentage of spermatozoa illustrating linear progressive motility (40 ± 9.7% vs. 70 ± 12.3%). However, no correlation was found between sperm chromatin condensation and morphology, count, and motility. This study suggests that chromatin condensation constitutes a valuable parameter in the assessment of male fertility, completely independent of conventional sperm parameters. Consequently, the inclusion of chromatin condensation to routine laboratory investigations of semen prior to assisted reproduction is strongly recommended.

**Keywords** sperm chromatin condensation, sperm count, sperm morphology, sperm motility

Address correspondence to Dr. M. E. Hammadeh, Department of Obstetrics and Gynaecology, University of Saarland, 66421 Homburg/Saar, Germany. E-mail: Frmham@med-rz.uni-sb.de

In spite of the numerous treatment regimens that have been employed over the years, medical treatment of male infertility has largely been a failure. However, thanks to the advent of assisted reproduction technology (ART), many subfertile men today have the chance of realizing their dream of biological fatherhood, the severity of their condition not withstanding. Semen analysis constitutes the most important investigation of male infertility [17], with sperm morphology, motility, and concentration representing the three most important factors in the assessment of male reproduction potential [16]. Although this holds true for natural conception, these parameters have not been proven to be equally important in ART. Therefore, there is still a need to develop more sensitive diagnostic techniques capable of identifying subfertile states that are amenable to the few therapeutic options available [1].

It has been postulated that the existence of subtle sperm abnormalities that are unrecognized by conventional semen analysis may explain reproduction failure in men [4]. Such structural or biochemical defects are thought to be associated with chromatin packaging in the sperm nucleus [22]. Poor chromatin packaging and possible DNA damage may contribute to failure of sperm decondensation and subsequently fertilization failure [18] or habitual abortion following fertilization [13]. Therefore, a greater understanding of the molecular basis of male infertility may be essential in broadening knowledge on the effect of abnormal spermatozoa on fertilization and embryo development [19].

The degree of chromatin condensation can be assessed with the aid of acidic aniline blue staining, which discriminates between lysine-rich histones and arginine- and cystein-rich protamines [12]. This technique gives a specific positive reaction for lysine and reveals differences in basic nuclear protein composition of ejaculated human spermatozoa. Histone-rich nuclei of immature spermatozoa are rich in lysine and will consequently take up the blue stain. On the other hand, protamine-rich nuclei of mature spermatozoa are rich in arginine and cysteine and contain relatively low lysine [5, 8] and will not be stained by aniline blue. This study was carried out to determine the value of sperm chromatin condensation in the assessment of male fertility potential.

## MATERIALS AND METHODS

A total of 165 semen samples were collected from 90 men attending our andrology and IVF laboratory for infertility disorders (cases) and 75 healthy donors (control). The samples were evaluated for chromatin condensation, sperm morphology, concentration, and linear progressive motility. Semen samples were obtained by masturbation following a 3–5-day sexual abstinence. Many smears were prepared before and after semen processing by the swim-up technique, air dried, and fixed for 30 min in 3% glutaraldehyde in phosphate-buffered saline for the assessment of morphology and nuclear maturity which was carried out by the aniline blue staining method [20].

The smear was stained for 5 min in 5% aqueous aniline blue solution (pH 3.5). Sperm heads containing immature nuclear chromatin stain blue (chromatin not condensed) and those with mature nuclei do not take up the stain (chromatin condensed). The percentage of spermatozoa stained with aniline blue was determined by counting 200 spermatozoa per slide under a bright-field illumination at a magnification of 100. Depending on the proportion of sperm head stained by aniline blue and using 25% as the cutoff point, samples were classified into two categories: good quality (unstained, chromatin condensed) and bad quality semen (stained,

chromatin uncondensed). Semen quality was evaluated according to WHO guidelines [21], except for morphology, which was evaluated according to strict criteria [15].

### Statistical Analysis

The statistical analysis was performed using the SPSS-PC package, including the calculation of the differences, standard deviations, significance of difference, as well as the Kruskal–Wallis test for nonparametric comparisons and the Mann–Whitney *U* test for two non-paired data. The Spearmann rank correlation coefficient was used to determine the correlation between two variables. The results are expressed as means ± standard deviations and a $p$ value of less than .05 was considered statistically significant.

## RESULTS

The mean age of the patients and controls was 34 ± 4.0 years (range 29–40). The mean percentage of morphologically normal spermatozoa ( according to strict criteria) was 12 ± 1.2% for the patients samples and 23.9 ± 1.9% for the control group ($p < .001$). The most frequent sperm abnormalities were represented by amorphous (35 ± 4.0%), microcephalic (11.7 ± 1.9%), and elongated spermatozoa (9.1 ± 1.7%), as well as spermatozoa with tail abnormalities (8 ± 1.2%). When classified according to the three main segments of a sperm cell, head abnormalities were most frequent (60 ± 3.2%), followed by tail (8 ± 1.2%) and midpiece abnormalities (4.7 ± 1.9%). A similar trend was observed in the control group.

A cutoff point of $20 \times 10^6$ mL [21] was used to distinguish between normal (count $\geq 20 \times 10^6$/mL) and subnormal semen (count $< 20 \times 10^6$/mL). Whereas only 50 out of the 90 patients (55%) had a normal sperm count, all the 75 (100%) had a normal sperm count in the control group. By comparing between the two groups a significant difference ($p < .001$) was observed. Similarly, a significant difference ($p < .001$) was shown between the patient samples and the control with regard to the percentage of spermatozoa in the ejaculate, illustrating linear progressive motility (40 ± 9.7% and 70 ± 12.3%, respectively). On the other hand, whereas only 55 ± 12.0% of the samples from the cases were unstained by aniline blue (chromatin condensed), 78 ± 19% of the samples in the control group did not take up the stain (chromatin condensed). A significant difference ($p < .001$) between the two groups was also observed. A correlation between chromatin condensation and morphology, count, and motility was demonstrated neither in the patients nor in the control group (Tables 1, 2).

**Table 1.** Comparison between cases and control with regard to morphology, count, motility, and chromatin condensation

| | Patient group % ($n = 90$) | Control group % ($n = 75$) | $p$ value |
|---|---|---|---|
| Normal morphology ≥14 % | 12.1 ± 1.2 | 23.9 ± 1.9 | .001 |
| Sperm count ≥ $20 \times 10^6$/mL | 55.5 | 100.0 | .001 |
| Linear progressive motility | 40.0 ± 9.7 | 70.0 ± 12.3 | .001 |
| Nuclear maturity (chromatin condensation) | 55.0 ± 12.0 | 78.0 ± 19.0 | .001 |

**Table 2.** Correlation between chromatin condensation and sperm morphology, count, and sperm motility in patients and control group

| | Patients ($n$ = 90) | | | | | | Control ($n$ = 75) | | |
|---|---|---|---|---|---|---|---|---|---|
| | Neat semen | | | Processed semen | | | Neat semen | | |
| | Morphology ≥14% | Count | Motility | Morphology ≥14% | Count | Motility | Morphology ≥14% | Count | Motility |
| Chromtin condensation | $r$ = .03<br>$p$ = .77 | $r$ = .09<br>$p$ = .43 | $r$ = .37<br>$p$ = .77 | $r$ = .07<br>$p$ = .52 | $r$ = .16<br>$p$ = .18 | $r$ = .27<br>$p$ = .51 | $r$ = .09<br>$p$ = .59 | $r$ = .41<br>$p$ = .91 | $r$ = .71<br>$p$ = .35 |

## DISCUSSION

In this study, a significant difference was found between patients and control group with regard to normal morphology of spermatozoa (12.1 ± 1.2% vs. 23.9 ± 1.9%, $p = .001$), chromatin condensation (55 ± 12% vs 78 ± 19.0%, $p = .001$), and motility (40 ± 9.7% vs. 70 ± 12.3%, $p = .001$) (Table 1). In spite of the divergence in viewpoints concerning the value of the different semen parameters in the assessment of male fertility, most authors are unanimous that sperm morphology, motility, and concentration represent the three most important factors. Although this holds true for natural conception, it has not proven to be equally so in assisted reproduction technology (ART); whether none of the above factors seem to play a major role in ICSI therapy [10]. The need for other sperm function tests with which male fertility can be better assessed and fertilization outcome predicted still constitutes a challenge in ART. In the present study no correlation was found between chromatin condensation (aniline blue test) and spermatozoa motility ($r = .37$, $p = .77$), count ($r = .09$, $p = .43$), and morphology ($r = .03$, $p = .08$) in either the patient groups or the control group ($r = .71$, $p = .35$, count $r = .41$, $p = .91$ and $r = .09$, $p = .59$, respectively) (Table 2).

An association between abnormal sperm chromatin condensation and male infertility has been described [3, 7]. Auger et al. [2] used aniline blue staining as a marker for sperm chromatin defects and recommended the addition of this parameter to routine semen analysis. Chromatin condensation is now accepted as one of the most important of the numerous sperm function tests [9]. In our previous study [11] we demonstrated that chromatin condensation visualized by aniline blue staining is a good predictor for IVF outcome. In a group of patients with 0–20% stained spermatozoa (G.1) the fertilization and pregnancy rates were higher than that in other groups (G.2) with >20% (79.9%, 52.8% vs. 58.8%, 29.5%, respectively). Semen assessment for sperm chromatin condensation may be particularly important in intracytoplasmic sperm injection (ICSI) where most of the natural selection mechanisms involved in the fertilization process are bypassed, thus raising some concerns about the well-being of children resulting from this therapy [6, 14]. The highly significant difference between semen parameters of patients and health donors with regard to chromatin condensation as well as sperm morphology, sperm count, and motility indicates that all of these factors are important in the determination of the fertility potential of the male. The absence of a correlation between chromatin condensation and these other sperm function tests further suggests that they are independent parameters from one another. Therefore, the current study strongly suggests that chromatin condensation constitutes a valuable parameter in the assessment of male fertility, completely independent of conventional semen parameters, and should be recommend to routine laboratory investigations of semen prior to assisted reproduction.

## REFERENCES

1. Aitken RJ (1983): Clinical assessment of human sperm function. J Obstet Gynecol 3:48.
2. Auger J, Mesbah M, Huber C, Dadoune JP (1990): Aniline blue staining as a marker of sperm chromatin defects associated with different semen characteristics discriminates between proven fertile and suspected infertile men. Int J Androl 13:452–462.
3. Bach O, Glander HJ, Scholz G, Schwarz J (1990): Electrophoretic patterns of spermatozoal nucleoproteins (NP) in fertile men and infertile patients and comparison with NP of somatic cells. Andrologia 22:217–224.

4. Bedford JM, Bent MJ, Calvin H (1973): Variations in the structural character and stability of the nuclear chromatin in morphologically normal human spermatozoa. J Reprod Fertil 333:19–28.
5. Calvin HI (1976): Comparative analysis of the nuclear basic proteins in rat, human, guinea pig, mouse and rabbit spermatozoa. Biochim Biophys Acta 434:377–389.
6. Diemer T, Desjardins C (1999): Developmental and genetic disorders in spermatogenesis. Hum Reprod Update 5:120–140.
7. Foresta C, Zorui M, Rossato M, Varotto A (1992): Sperm nuclear instability and staining with aniline blue: abnormal persistence of histones in spermatozoa in fertile men. Int J. Androl 15:330–337.
8. Gusse M, Sautiere P, Belaiche D (1986): Purification and characterisation of nuclear basic proteins of human sperm. Biochim Biophys Acta 884:124–134.
9. Haidl G (1996): Andrologische Diagnostik Heute. Fortschr Med. 114:465–469.
10. Hammadeh ME, Al-Hasani S, Doerr S, Stieber M, Rosenbaum P, Schmidt W, Diedrich K (1999): Comparison between chromatin condensation and morphology from testis biopsy extracted and ejaculated spermatozoa and their relationship to ICSI outcome. Hum Reprod 2:363–367.
11. Hammadeh ME, Stieber M, Haidl G, Schmidt W (1998): Association between sperm cell chromatin condensation, morphology based on strict criteria and fertilization, cleavage, and pregnancy rates in IVF programme. Andrologia 30:29–35.
12. Hofmann N, Hilschler B (1991): Use of aniline blue to assess chromatin condensation in morphologically normal spermatozoa in normal and infertile men. Hum. Reprod 6:979–982.
13. Ibrahim ME, Moussa MAA, Dedersen H (1988): Sperm chromatin heterogeneity as an infertility factor. Arch Androl 21:129–133.
14. Johnson MD (1998): Genetic risks of intracytoplasmic sperm injection in the treatment of male infertility: recommendations for genetic counselling and screening. Fertil Steril 70:397–411.
15. Kruger TF, Menkveld R, Stander FSH (1986): Sperm morphology features as a prognostic factor in IVF. Fertil Steril 46:1118–1123.
16. Liu DY, Du-Plessisy P, Nayudu PL, Johnston WI, Baker HW (1988): The use of in vitro fertilisation to evaluate putative tests of human sperm function. Fertil Steril 49:272–277.
17. Ombelet W, Pollet H, Bosmans E, Vereecken A (1997): Results of a questionnaire on sperm morphology assessment. Hum Reprod 12:1015-1020.
18. Sakkas D, Urner F, Bianchi PG, Bizzaro D, Wagner T, Jacquenoud N, Manicardi G, Campana A (1996): Sperm chromatin anomalies can influence decondensation after intracytoplasmic sperm injection. Hum Reprod 11:837–843.
19. Sakkas D, Urner F, Bizzaro D, Minicardi G, Bianchi PG, Shoukir Y, Campana A (1999): Sperm nuclear DNA damage and altered chromatin structure: effect on fertilisation and embryo development. Hum Reprod 13:11–19.
20. Terquem A, Dadoune JP (1983): Aniline blue staining of human sperm chromatin: evaluation of nuclear maturation. In: The Cell. Andre J (Ed). London: Martinus Nijhoff, pp 249–252.
21. World Health Organisation (WHO) (1992): Laboratory Manual for the Examination of Human Semen and Semen Cervical Mucus Interaction. Cambridge, UK: Cambridge University Press, pp 6–7.
22. Zamboni L (1992): Sperm structure and its relevance to infertility. Arch Pathol Lab Med 116:325–344.