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Correlation of Sperm Nuclear Chromatin Condensation Staining Method with Semen Parameters and Sperm Functional Tests in Patients with Spinal Cord Injury, Varicocele, and Idiopathic Infertility

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ABSTRACT

Introduction: Our aim was to investigate sperm nuclear chromatin condensation and its correlation with semen parameters and vitality test in infertile patients with spinal cord injury (SCI), varicocele, and idiopathic infertility.

Materials and Methods: Sperm chromatin condensation was determined by aniline blue staining in 22 SCI-injured infertile men, 20 with varicocele, and 28 with idiopathic infertility. The results were compared with the semen analysis parameters and the hypo-osmotic swelling test results. Three grades of staining for sperm heads were distinguished: unstained, showing sperm maturity (G0); partially stained (G1); and completely stained, showing sperm immaturity (G2). The total score was calculated as: (G0 × 0) + (G1 × 1) + (G2 × 2).

Results: In all groups, the total staining score was higher than 75%, corresponding to a high degree of immaturity of sperm. Patients with SCI had a less sperm nuclear chromatin condensation and chromatin stability than patients with idiopathic infertility and varicocele (total scores, 98% versus 89% and 88%, respectively; $P < .01$). All of the patients had normal hypo-osmotic swelling test results. Sperm counts for all patients were within the reference range. The mean percentages for normal motility and morphology of the sperm were 15.5% and 15% for patients with SCI, 43% and 15% for patients with varicocele, and 62.5% and 54% for patients with idiopathic infertility. There was no correlation between sperm nuclear chromatin condensation and semen analysis parameters.

Conclusion: Aniline blue staining for sperm nuclear chromatin condensation is a method independent of semen analysis and demonstrates the internal structural defects of sperm. This method may have a predictive value in assessing fertility.

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Introduction

Assessing sperm morphology, motility, and concentration is the best way to investigate male fertility. These are the 3 most important factors in male reproduction potential.[1,2] However, these parameters have not been proven useful in predicting the results of assisted reproductive technology (ART).[3] Therefore, more sensitive diagnostic techniques to identify subfertility amenable to the therapeutic options must be developed,[4] especially in patients with idiopathic infertility, blood vessels diseases, and nervous system disorders.

Bedford and colleagues have stated that the existence of subtle sperm abnormalities that are unrecognized by conventional semen analyses may explain reproduction failures.[5] Such structural or biochemical defects are thought to be associated with chromatin packaging in the sperm nucleus.[5] Poor chromatin packaging and possible DNA damage may contribute to a failure of sperm decondensation and subsequently, fertilization failure or habitual abortion following fertilization.[6,7] The degree of chromatin condensation can be assessed with acidic aniline blue staining, which discriminates lysine-rich histones from arginine-rich and cysteine-rich protamines.[8] Histone-rich nuclei of immature spermatozoa are rich in lysine and consequently, take up the blue stain. Conversely, protamine-rich nuclei of mature spermatozoa are rich in arginine and cysteine, and contain relatively low amounts of lysine[9,10]; thus, they do not stain with aniline blue. Accordingly, evaluation of sperm chromatin condensation may be a good predictor of ART results.

This study was carried out to compare the results of sperm chromatin condensation with semen analysis parameters to assess male infertility in patients with spinal cord injury (SCI), varicocele, and idiopathic infertilities, and to determine the influence of external structure and parameters on the internal structure of sperm cells.

Materials and Methods

This prospective study was performed on infertile men who had been referred to Sina Hospital, Mirza Kouchak Khan Hospital, and Koassar Fertility and Impotency Center, in Tehran, Iran. From September 2002 to September 2003, 22 patients with SCI, 20 with varicocele, and 28 with idiopathic infertility and their partners were enrolled in the study. Informed consent was obtained from the subjects and their partners. To confirm the diagnoses, clinical and paraclinical assessments (hormone profile, antisperm antibodies, and ultrasonography) were performed in all 3 patient groups. The patients' partners also were examined to rule out female-factor infertility.

Group 1 included 3 patients with thoracic SCI (T1 to T10) and 19 patients with lumbar SCI (T10 to L2). All of these patients had a complete SCI and a history of paralysis of between 11 and 18 years' duration. In group 2, diagnosis of varicocele was made by physical examination and Doppler ultrasonography. Group 3 was composed of 28 infertile men with normal semen analysis results according to the criteria of the World Health Organization guidelines for semen analysis and Kruger and colleagues' strict criteria (volume, > 2 mL; pH, 7.2 to 7.8; sperm count, > 20 × 106/mL; sperm motility, > 25%; sperm morphology, > 14%),[11,12] and no diseases were identified in clinical and paraclinical assessments (hormone profile, antisperm antibodies, ultrasonography), corresponding to men with idiopathic infertility.

In all patients, semen samples were obtained in an antegrade fashion. In SCI patients, the bladder was emptied with a catheter first. Then, a Seager Model 14 Electroejaculator (Dalzell Medical System, The Plains, VA, USA) was used, and the semen was obtained into a sterile container. Semen volume, density, and pH; sperm count, morphology, and motility; and white blood cell count in the semen were determined according to the World Health Organization guidelines for semen analysis.[11] Sperm count was measured in a grade pattern using a Neubauer hemocytometer. Sperm motility was defined as the percentage of sperm demonstrating flagellar motion, categorized as A (significant rapid progressive motility), B (slow progressive motility), C (nonprogressive motility), and D (no motility). Sperm morphology was interpreted by the strict criteria defined by Kruger and colleagues.[12]

The degree of chromatin condensation was assessed using staining with aniline blue as previously described.[13] After sperm preparation, 5 μL of prepared spermatozoa was spread onto a glass slide and air-dried. Smears were fixed in 3% buffered glutaraldehyde in phosphate buffered

saline for 30 minutes. Slides were then stained with 5% aqueous aniline blue mixed with 4% acetic acid (pH, 3.5) for 5 minutes. A total of 100 to 200 sperm cells were evaluated, and the percentage of stained sperm heads was calculated. Three grades of staining for sperm heads were identified: unstained, showing sperm maturity (G0); partially stained (G1); and completely stained, showing sperm immaturity (G2). The total score was calculated according to the number of sperm heads with any of the 3 grades of staining by this formula: (G0 × 0) + (G1 × 1) + (G2 × 2).[14]

The hypo-osmotic swelling (HOS) test was performed in all 3 groups to evaluate sperm vitality and sperm wall integrity. One aliquot of 0.1 mL liquefied semen was added to 1 mL of hypo-osmotic solution. After incubation for 60 minutes at 37°C, samples were examined by a single technician using a phase-contrast microscope. The score was defined based on the 7 morphologic types of tail swelling (a, no change; b-g, various types of tail swellings). The HOS test was considered abnormal for a semen sample if fewer than 50% of the spermatozoa were swollen.[11]

Collected data were compared between the 3 groups of patients using a 1-way analysis of variance and the Tukey test. The Pearson correlation coefficient was used to study the relationship between semen parameters and sperm chromatin nuclear staining, and between semen parameters and HOS test types.

Results

Mean ages of men with SCI (group 1), varicocele (group 2), and idiopathic infertility (group 3) were 34 ± 4.6 years, 32 ± 2.2 years, and 34 ± 1.6 years. The mean age of their partners was 30.7 ± 2.3 years, and no cause of infertility was found in them.

The results of semen analyses for all patients are summarized in Table 1. The sperm count was within the reference range for the patients of the 3 groups, but the mean sperm count was higher for the patients in group 1 than it was for patients in groups 2 and 3 ($P < .05$). The mean percentage of sperm with normal motility was the lowest in patients in group 1 and highest in patients in group 3 ($P < .05$). The mean percentage of sperm with a normal morphology was significantly higher in patients in group 3 ($P < .01$). The seminal fluid pH was significantly higher in men in group 2 than it was for men in group 3 ($P < .05$). The mean number of white blood cells in the seminal fluid of patients in group 1 was significantly higher than it was in patients in groups 2 and 3 ($P < .01$). The other seminal fluid components and sperm parameters were not significantly different between patients of the 3 groups.

The total score of chromatin staining (representing sperm immaturity) in all 3 groups was higher than 75% (Table 2). No correlation was found between chromatin condensation and sperm parameters (Table 3). Patients in group 1 had lower chromatin condensation and stability (higher total score) when compared with patients in groups 2 and 3 . The level of G0 chromatin condensation was significantly lower in patients in group 1 when compared with patients in the other 2 groups ($P < .01$), while the level of G1 chromatin was higher in patients in group 1 than it was in patients in group 2 ($P < .01$); however, this level was not significantly higher than the level in patients in group 3. No significant differences were seen in the level of G2 between the 3 groups. Results of the HOS test were

TABLE 1. *Results of seminal fluid and sperm parameters in the 3 groups of patients*

	Number of patients	Volume (mL)	pH	Sperm count ($\times 10^6$/mL)	Sperm motility (%)	Sperm morphology (%)	White blood cells ($\times 10^6$/mL)
Group 1 (spinal cord injury)	22	2.5 ± 1.87	7.74 ± 0.26	110.11 ± 88.48†	15.63 ± 11.99†‡	15.06 ± 10.21	31.95 ± 21.78†‡
Group 2 (varicocele)	20	3.21 ± 1.64	8.01 ± 0.60*	56.1 ± 44.09*	43.1 ± 21.33*‡	15.45 ± 11.51	8.6 ± 13.02‡
Group 3 (idiopathic infertility)	28	3.42 ±1.87	7.55± 0.29*	78.92 ± 40.14*†	62.57 ± 12.66*†	53.93 ± 16.65	5.39 ± 11.21†

*significant difference between the patients of groups 2 and 3 (P < .05)
†significant difference between the patients of groups 1 and 3 (P < .001)
‡significant difference between the patients of groups 1 and 2 (P < .01)

Table 2. *Nuclear maturity and chromatin condensation categories in the 3 groups of patients*

	G0 (%)	G1 (%)	G2 (%)	Total score (%)
Group 1 (spinal cord injury)	11.04 ± 5.31*†	78.68 ± 9.55†	9.59 ± 5.55	97.86 ± 50.59*†
Group 2 (varicocele)	22.25 ± 8.08†	67.20 ± 10.52†	11.05 ± 7.45	89.30 ± 12.16†
Group 3 (idiopathic infertility)	19 ± 11.98*	74.1 ± 13.41	6.89 ± 6.40	87.82 ± 13.74*

G0: not stained showing sperm maturity, G1: partially stained, G2: completely stained showing sperm immaturity, Total score = (G0 × 0) + (G1 × 1) + (G2 × 2)
*significant difference between the patients of groups 1 and 3 (P < .01)
†significant difference between the patients of groups 1 and 2 (P < .01)

Table 3. *Correlation between sperm chromatin condensation and sperm morphology, count, and motility, in the 3 groups of patients*

	Groups	Pearson r (*P* value)		
		Sperm morphology	Sperm count	Sperm motility
Chromatin Condensation (total score)	Group 1 (spinal cord injury	0.07 (.52)	0.16 (.18)	0.27 (.51)
	Group 2 (varicocele	0.03 (.77)	0.09 (.43)	0.37 (.77)
	Group 3 (idiopathic infertility	0.09 (.59)	0.41(.91)	0.35 (.71)

normal in all patients (less than 50% type a spermatozoa). Significant differences were found between the 3 groups, especially with regard to some types of tail defects (Table 4). Type a was more frequent in patients in group 3. Differences also were significant in types c, d, and g. In the patients of group 2, seminal pH had a inverse relationship with tail defects, although this relationship was not significant. In the patients of group 2, seminal pH had a direct relation with sperm motility and a inverse relation with tail defects ($P < .05$).

Discussion

As indicated by WHO protocol, the high prevalence of a male factor for infertility mandates a complete andrologic examination that must include functional sperm parameters and an HOS test.[11] In this study, a significant difference was found between sperm parameters in patients with SCI, varicocele, and idiopathic infertility, especially with regard to sperm count, motility, and morphology. These 3 factors are important, but none of them seem to play a major role in the outcome of intracytoplasmic sperm injection (ICSI).[13] Using other sperm functional tests, such as chromatin condensation, is still under debate.[15,16] In this study, using aniline blue staining, a relatively high proportion of immature sperm was found, while no correlation was found between chromatin condensation (aniline blue staining) and semen analysis. The HOS test results and most semen analysis parameters were normal in all patients. Thus, semen analyses and

Table 4. *Results of HOS test in the 3 groups of patients*

	Type *a* (%)	Type *b* (%)	Type *c* (%)	Type *d* (%)	Type *e* (%)	Type *f* (%)	Type *g* (%)
Group 1 (spinal cord injury	11.45 ± 1.14*†	21.86 ± 2	11.72 ± 1.69*	5.4 ± 1.68*†	13.81 ± 1.56	13.4 ± 1.36	21.45 ± 1.26†
Group 2 (varicocele	13.7 ± 2.38†	22.6 ± 1.78	12.9 ± 1.65	3.7 ± 1.8†	12.25 ± 1.74	12.3 ± 1.65	23.35 ± 3.06†
Group 3 (idiopathic infertility	14.35 ± 1.68*	23.10 ± 1.79	13.10 ± 1.89*	2.96 ± 1.57*	12.64 ± 1.63	12.32 ± 1.74	22.89 ± 1.85

*significant difference between the patients of groups 1 and 3 (P < .05)
†significant difference between the patients of groups 1 and 2 (P < .05)

the HOS test failed to show any defect that might impact the results of ICSI. The association of abnormal sperm chromatin condensation with male infertility has been previously described.[6,13,15] Consequently, sperm chromatin condensation may indicate a defect in the sperm of infertile men with normal semen analyses that may lead to ICSI failure.

Some authors have investigated aniline blue staining as a marker for sperm chromatin defects to predict ART results. Haidl and Schill have found a strong correlation between normal chromatin condensation and fertilization rate in in vitro fertilization (IVF). They have recommended that this method be performed prior to IVF because of the significant correlation between fertilization rate of spermatozoa in IVF and normal chromatin condensation.[7] However, Hammadeh and colleagues have found different results in their studies.[13,17-19] In a study in 1996, they demonstrated that chromatin condensation has no predictive value for ICSI outcomes. They compared the outcome of ICSI between patients with 0% to 29% stained spermatozoa with aniline blue and those with more than 29% stained spermatozoa and found pregnancy rates of 18.5% and 35.5%, respectively.[13] In another study of 96 infertile men, they compared the fertilization rate after IVF between men with 20% or fewer stained spermatozoa and those with more than 20%. The fertilization rates were 79.9% versus 58.8%, respectively.[17] Hammadeh and colleagues performed research similar to ours and found no correlation between sperm morphology, chromatin condensation, and sperm count either in the fresh or in the processed semen samples.[18] Later, in 2001, these authors designed a case-control study to determine the value of sperm chromatin condensation to assess male fertility. A total of 165 semen samples from 90 patients and 75 healthy donors (control) were examined for chromatin condensation by aniline blue staining. A lower percentage of the samples from infertile patients was unstained by aniline blue ($P < .001$). However, no correlation was found between sperm chromatin condensation and morphology, count, and motility. The authors concluded that chromatin condensation constitutes a valuable parameter in assessing male fertility, independent of conventional sperm parameters. The inclusion of chromatin condensation in routine laboratory investigations of semen prior to assisted reproduction is strongly recommended by this group.[19]

Sperm chromatin packaging quality has been assessed by the chromomycin A3 (CMA3) fluorochrome and the presence of DNA damage in spermatozoa, using in situ nick translation, as well. Sakkas and coworkers have shown that normal men present sperm parameters with a normal morphology of > 20%, CMA3 fluorescence of < 30%, and exhibit endogenous nicks in < 10% of their spermatozoa. When they separated patients according to these values, no difference was observed in fertilization rates after ICSI. When the unfertilized ICSI oocytes were examined, they found that patients with CMA3 fluorescence of < 30% and nicks in < 10% of their spermatozoa had only 21.6% of their unfertilized oocytes containing spermatozoa that remained condensed. In contrast, patients with higher CMA3 and nick values had a significantly higher percentage, 48.9%, of their unfertilized oocytes containing condensed spermatozoa. Sperm morphology showed no such pattern. The percentage of spermatozoa that had initiated decondensation in unfertilized oocytes was not influenced by morphology, CMA3 fluorescence, or nicks. They postulated that poor chromatin packaging and/or damaged DNA may contribute to failure of sperm decondensation after ICSI and result in failure of fertilization.[3]

Controversial reports on the practical use of sperm nuclear condensation assessment warrant further studies. However, it seems that semen assessment for sperm chromatin condensation may be useful in the ICSI procedure,[3] where much of the natural selection mechanism involved in fertilization is bypassed. The absence of a correlation between chromatin condensation and sperm functional tests in our results indicates that they are independent parameters. The last step of fertilization is determined by chromatin decondensation in spermatozoa, in which the histones are replaced by protamine.[3] The ratio of replacement can be determined by aniline blue staining or other methods and may be of good prognostic value in male fertility.

Conclusion

Aniline blue staining for chromatin condensation of sperm nuclear is an independent factor from sperm functional parameters. Thus, it may be a good means of assessing the fertilization potential by demonstrating internal structural defects in the fertility potential of

sperm. We recommend that it be included in routine laboratory investigations of semen prior to assisted reproduction. However, the efficacy of this test must be evaluated by further studies in the future.

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