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# Comparative Study of Cytochemical Tests for Sperm Chromatin Integrity

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**ABSTRACT:** Tests were carried out on sperm from 40 fertile and infertile men to evaluate 2 DNA in situ denaturation methods using acridine orange (AO; the modified Rigler-Roschlau method and the Tejada method), alongside routine aniline blue (AB) and toluidine blue (TB) tests in our modification, and in order to estimate and compare the practical value of different in situ cytochemical tests for sperm chromatin structure. In addition, the methods were applied to rat and boar spermiogenesis models. The sperm heads with abnormal versus normal chromatin structure were specified as orange-red versus green by the AO method, blue versus uncolored by the AB method, and purple-violet versus light blue by the TB method. A good correlation for the proportion of sperm heads with abnormal chromatin structure was found among all the methods ($r = .63–.70$; $P < .01$), which characterized all 4 techniques as sensitive enough to estimate in situ sperm DNA integrity. In our study, the average value of abnormal cells was 17% ± 3.8% and 30.2% ± 6.8% for the fertile and infertile groups of men, respectively, setting a threshold of 95% probability at 23% as judged by the Rigler-Roschlau method. This compared with 23.9% ± 7.5% and 52.1% ± 20.8% ($P \leq .05$) for the fertile and infertile groups, respectively, setting a threshold at 31%, as judged by the Tejada method. The technical advantages and disadvantages of each method are briefly reported.

Key words: Fertility, DNA normality, sperm maturation.

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The nuclear status of human sperm cells has great importance in fertility (Tejada et al, 1984; Ibrahim et al, 1988; Dadoune, 1995; Hoshi et al, 1996; Samochabone et al, 1998; Spano et al, 2000). It has been recently argued that the high percentage of spermatozoa with impaired DNA structure in ejaculates is predictive of subfertility and infertility, however, the high percentage of spermatozoa with normal DNA is not a direct predictor of good fertility potential because many factors (eg, sperm concentration, motility, etc) are involved in the fertilization process (Evenson et al, 1999). The integrity of sperm chromatin structure results from the influence of endogenous factors, including sperm maturation, and from exogenous factors, such as peroxidative damage of DNA, which may arise from infectious or toxic agents (Irvine et al, 2000).

Sperm maturation takes place in round and elongating spermatids in the testis and is completed in the epididymis. It is generally believed that the main event of maturation that affects the chromatin structure and its staining properties depends on the proper replacement of histones by transition proteins and then by more basic protamines. This provides the final, very compact packaging of the DNA, which is enforced by cross-linking of protamine disulfide bonds.

It was found that in abnormal human sperm, histones may be partially or entirely replaced by protamines and that the chromatin remains loosely packed, which can be shown by the aniline blue (AB) test (Dadoune et al, 1988).

The changes in the secondary DNA structure during normal spermatogenesis have not been extensively studied. Meiotic crossing-over is associated with the genetically programmed introduction of DNA double-strand breaks (Smith and Nicolas, 1998). Many germ cells are sorted during spermatogenesis by apoptosis (Blanco-Rodriguez, 1998). Studies performed on rats by Stephan et al (1996) revealed DNAse 1 transcription in some spermatogonia and in all spermatids even after they reached the epididymis. However, nick-translation, performed by these authors on paraffin sections revealed positive cells only among a few spermatogonia.

As tested by the in situ DNA denaturation test with acridine orange (AO; Darzynkiewicz et al, 1975; Darzynkiewicz, 1994), it was shown that DNA is more prone to denaturation by heat or low pH in human sperm nuclei with abnormal chromatin structure than in nuclei with normal chromatin. This test has been applied in flow cytometry as the sperm chromatin structural assay (SCSA) for the detection of male sperm quality (Evenson, 1980).

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The chromatin proteins in sperm nuclei with impaired DNA appeared to be more accessible to binding with the acidic dye, as found by the AB test (Auger et al, 1990) and by the correlative results between the AO and AB tests (Liu and Baker, 1992). In turn, it can be logically inferred that the phosphate residues of the DNA in such nuclei will be more liable to binding with basic dyes. Such conclusions also come from the results of staining with basic dyes, such as toluidine blue (TB), methyl green, Giemsa stain, 4′,6′-diamidino-2-phenylindole (DAPI), which have been reported by some authors (Mello, 1982; Bianchi et al, 1993; Andreetta et al, 1995). Thus, the chromatin structural probes using nuclear dyes are sensitive and simple in their performance and attractive for practical use. Their cytochemical grounds are, however, rather complex (analyzed in more detail in the appendix).

To estimate and compare the practical value of different in situ cytochemical tests, 2 DNA denaturation methods using AO were used, one combined from the earlier recommendations of Roschlau (Roschlau, 1965) and Rigler (Rigler, 1966; the RRAO method) and used earlier for in situ detection of apoptotic cells (Erenpreisa et al, 1997), and the other, suggested by Tejada et al (1984; the TAO method), for sperm cells. In addition, the well-known test with acidic AB (Terquem and Dadoune, 1983) and staining with basic TB by our own method, which is similar to the one recently proposed by Andreetta et al (1995), were carried out on the same human sperm material. The material was obtained from donors and patients from childless couples. In addition, the applied methods were characterized for their adequacy using the model of rat and boar spermatogenesis.

## Methods

Human sperm samples from 40 men were used in this study. Twenty-six men were healthy and fertile, 14 were subfertile or infertile patients (semen analysis showed different degrees of oligozoospermia, teratozoospermia, or asthenoteratozoospermia in childless couples in whom a female factor had been excluded). Samples from the 40 men were studied by all 4 methods; however, 60 samples were examined by the RRAO method. The additional 20 samples consisted of specimens from 5 fertile and 15 infertile or subfertile men. All samples were evaluated for standard semen parameters according to World Health Organization criteria (WHO, 1992). For comparative purposes, imprints of rat and boar testis and epididymis were also examined.

### Staining Protocols

Staining protocols were applied after 30 minutes of semen liquefaction at 37°C and after sperm smears had been air-dried for 1 hour.

To detect the percentage of immature sperm cells in human material, 300 cells were estimated in each sample using a Nikon TE 300 fluorescence microscope (Nikon GmbH, Düsseldorf, Germany). Excitation was achieved with a mercury lamp and use of blue filters.

Statistical analysis was performed with Student's *t*-test.

### Acridine Orange Staining

*Modified Rigler-Roschlau Method*—The RRAO method was reported by Erenpreisa et al (1997). Air-dried smears were fixed in freshly made 96% ethanol-acetone (1:1) at 4°C for 30 minutes up to a maximum of 24 hours. Smears were rehydrated in 96% ethanol for 5 minutes, in 70°C ethanol for 5 minutes, and 30% ethanol for 3 minutes at room temperature. Smears were treated with phosphate buffered saline (PBS) for 5 minutes followed by treatment with 1 N HCl for 1 minute at 60°C, rinsed 3 times in distilled water for 2 minutes each, and dipped into McIlvain citric phosphate buffer (pH 4.0) for 5 minutes. Smears were stained for 15 minutes with AO (0.038 mg/mL; $10^{-4}$ M) diluted in McIlvain buffer (pH 4.0), which had been prepared daily from a stock solution consisting of 7.6 mg AO (Sigma Chemical Company, St Louis, Mo) in 1 mL distilled water. Slides were rinsed 3 times for 5 minutes each with AO $10^{-6}$ M in the same buffer. Slides were covered with a coverslip before the slides could dry, sealed with nail polish, and stored in the dark at 4°C.

Sperm cell heads with good DNA integrity had green fluorescence, and those with diminished DNA integrity had orange-red staining. Samples could be scored within 48 hours after staining.

*TAO Method*—The TAO method was reported by Tejada et al (1984). Air-dried smears were fixed overnight in methanol-glacial acetic acid (3:1) at room temperature. The slides were removed from the fixative and allowed to dry for a few minutes before staining with AO (0.19 mg/mL, pH 2.5) for 5 minutes at room temperature. Staining solution was prepared daily from a stock solution consisting of 1 mg AO (Sigma) in 1000 mL of distilled water and stored in the dark at 4°C. To prepare the staining solution, 10 mL of the stock solution was added to 40 mL of 0.1 M citric acid and 2.5 mL of 0.3 M $Na_2HPO_4 \cdot 7H_2O$. All solutions were maintained at room temperature. After staining the slides were gently rinsed in a stream of distilled water and sealed under a coverslip with nail polish.

Sperm cell heads with good DNA integrity had green fluorescence, and those with diminished DNA integrity had orange-red staining. Samples could be scored within 1 hour after staining.

### Aniline Blue Staining

This method was reported by Terquem and Dadoune (1983). Air-dried smears were fixed in 3% glutaraldehyde in PBS for 30 minutes, dipped twice in PBS for 5 minutes, stained with AB (pH 3.5) for 7 minutes, washed with PBS, and air-dried. Staining solution was prepared daily with 5 g AB per 100 mL PBS, boiled for a short while, filtered, and adjusted to a pH of 3.5 with glacial acetic acid.

Sperm cell heads with good chromatin integrity were nearly colorless, and those with diminished integrity were blue.

### Toluidine Blue Staining

Air-dried smears were fixed in freshly made 96% ethanol-acetone (1:1) at 4°C for 30 minutes, hydrolyzed in 0.1 N HCl at 4°C for 5 minutes, and rinsed 3 times in distilled water for 2 minutes each. Smears were stained with 0.05% TB (Merck,

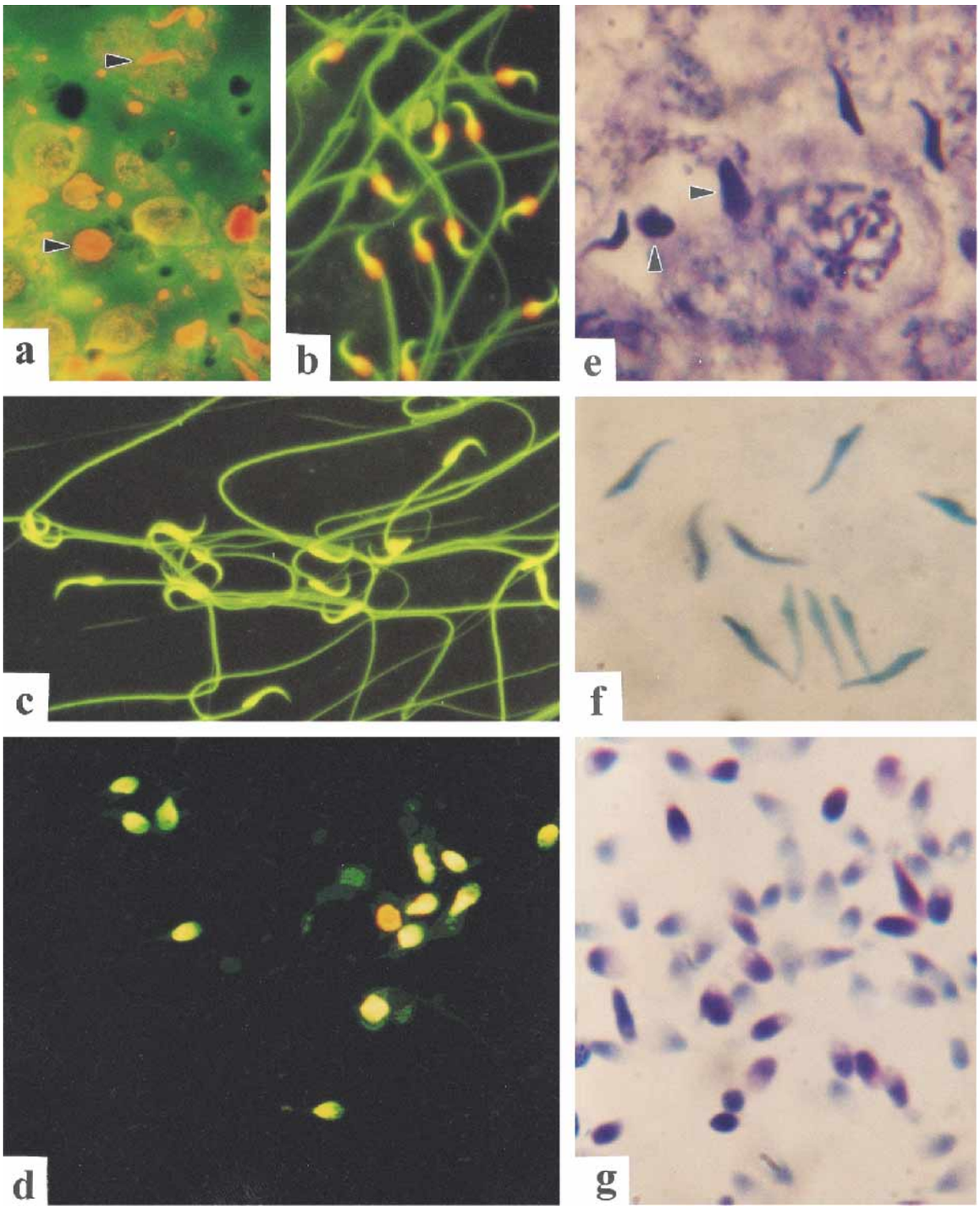


Figure 1. Change in the staining (fluorescence) of the sperm heads depending on maturity in rat testis and epididymis **(a–c, e, f)** and in human ejaculate **(d, g)**. Stained with AO (Rigler-Roschlau method): **(a)**, round and elongated spermatids in rat testis are orange-red (arrowheads); **(b)** sperm maturation in caput epididymis seen as double-colored sperm heads; **(c)** mature spermatozoa in cauda epididymis, sperm heads are green (yellowish); **(d)** human ejaculate, mature sperm heads are green, immature are orange-red. Stained with TB: **(e)** round and elongated spermatids in rat testis are violet; **(f)** sperm maturation in caput epididymis seen as light blue and dark blue spermatids; **(g)** human ejaculate, mature sperm heads are light blue, immature are violet. Magnification: **(a–c)**, ×1500; **(d)**, ×2500; **(e–g)** ×3200.

*Percentage of immature sperm cells in fertile and infertile men with RRAO and TAO methods**

| Immature Sperm Cells, % (Tejada's Method) | | Immature Sperm Cells, % (Rigler-Roschlau Method) | |
|---|---|---|---|
| Fertile Men | Infertile Men | Fertile Men | Infertile Men |
| 33 | 50 | 16 | 54 |
| 22 | 59 | 10 | 33 |
| 31 | 27 | 17 | 21 |
| 21 | 56 | 9 | 7 |
| 19 | 98 | 28 | 43 |
| 16 | 44 | 7 | 28 |
| 19 | 58 | 16 | 37 |
| 17 | 21 | 6 | 21 |
| 19 | 56 | 16 | 44 |
| 54 | 45 | 28 | 25 |
| 37 | 59 | 21 | 39 |
| 10 | 35 | 5 | 20 |
| 25 | 67 | 21 | 56 |
| 25 | 54 | 6 | 25 |
| 22 | | 8 | 14 |
| 19 | | 14 | 19 |
| 22 | | 21 | 25 |
| 25 | | 17 | 19 |
| 18 | | 18 | 92 |
| 28 | | 14 | 87 |
| 23 | | 15 | 14 |
| 22 | | 25 | 16 |
| 23 | | 17 | 11 |
| 22 | | 33 | 19 |
| 24 | | 28 | 26 |
| 25 | | 25 | 13 |
| | | 9 | 21 |
| 23.9 ± 7.5 | 52.1 ± 21 | 12 | 13 |
| | | 19 | 34 |
| Threshold 31% ($P \leq .05$) | | 7 | |
| | | 25 | |
| | | 17 ± 3.8 | 30.2 ± 6.8 |
| | | Threshold 23% ($P \leq .05$) | |

* RRAO indicates the recommendations of Roschlau and Rigler; TAO, Tejada's methods.

Poole, Dorset, United Kingdom) for 10 minutes. The staining buffer consisted of 50% citrate phosphate (McIlvain buffer, pH 3.5). Permanent preparations were dehydrated in tertiary butanol twice for 3 minutes each at 37°C and in Histoclear twice for 3 minutes each, followed by embedding in DPX.

Sperm cell heads with good chromatin integrity were light blue; those of diminished integrity were deep violet (purple).

## Results

The nuclei of round spermatids in rat and boar testes were orange-red by both AO methods (Figure 1a), blue by the AB method (not shown), and deep violet by the TB method (Figure 1e). During elongation, the spermatids acquired another color, which changed through a double-color stage from the anterior to posterior pole, from red to green by both AO techniques (Figure 1b), from blue to colorless by AB the technique (not shown), and from purple to light blue by the AB method (Figure 1f). The proportion of rat sperm cells with changed color was greater in caput epididymides and reached 100% in cauda epididymides that contained only mature sperm. The boar sperm heads in epididymides were all green; bicolored spermatids were observed in testes only.

All 4 methods exhibited a similar average proportion of sperm cells with abnormal chromatin structure in studied human material, respectively, for the following methods: 24.9% ± 4.8% for TB, 21.2% ± 3.8% for RRAO, 29.1% ± 3.7% for AB, and 31.8% ± 3.6% for TAO ($P \leq .05$), determined for 40 samples from fertile and infertile men (Table).

A good correlation was found in this study between the 2 AO in situ DNA denaturation methods ($r = .67$, $P < .01$; Figure 2), however, the absolute values of the proportion of red (or orange) sperm heads using the TAO method were on average 10% higher than they were with

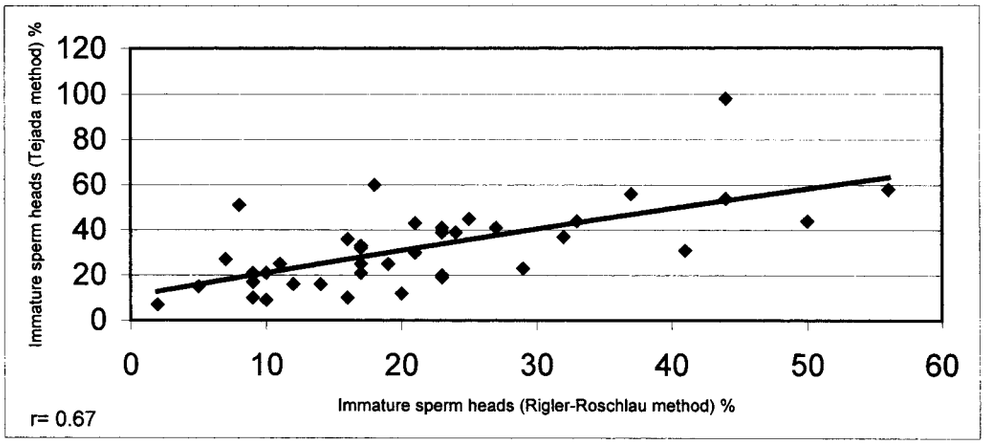


Figure 2. Relationship between the percentage of immature sperm cells revealed by the RRAO and TAO methods in 40 human ejaculate samples.

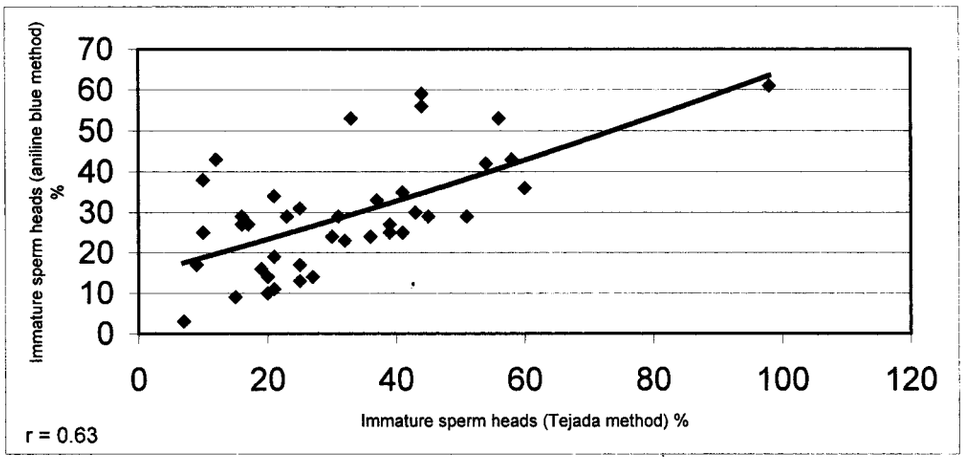


Figure 3. Relationship between the percentage of immature sperm cells revealed by the TAO and AB methods in 40 human ejaculate samples.

the modified RRAO technique. Because they were embedded in water, the preparations faded quickly, and by the next day the fluorescent colors had lost their clarity. By contrast, specimens that had been stained with the modified RRAO technique and embedded in the diluted buffered fluorochrome retained a good fluorescent pattern for 48 hours if they were kept refrigerated in a dark polyethylene bag. The repeated readings of the proportion of the cells with impaired DNA did not vary much during this time (an average variation coefficient of 10% when checked in a special experiment on 6 semen samples; not shown).

Aniline Blue staining also showed good correlation with both AO methods ($r = .63$, $P < .01$ for TAO and $r = .68$, $P < .01$ for the modified RRAO method; Figures 3 and 4). However, in our hands, the AB method seemed very dependable on the basis of the thickness of a smear, and ''cooperative'' effects (all cells stained or all unstained) were often found in thick places (these places were not used in our counts). AB and TB staining results were highly correlative ($r = .85$, $P < .01$; Figure 5).

Toluidine blue staining (purple-violet heads) showed a good correlation with counts of red-orange sperm heads as revealed through both AO methods ($r = .70$ and $r = .67$, $P < .01$ for the TAO and RRAO methods, respectively; Figures 6 and 7). The absolute values of deep violet heads were closer to the absolute values of red-orange heads obtained through the RRAO modification rather than that of the TAO method with AO (24.9% ± 4.8%, 21.2% ± 3.8%, and 31.8% ± 3.6%, $P \leq .05$ for the TB, RRAO, and TAO methods, respectively). In turn, the absolute values of blue heads obtained through the AB method (29.1% ± 3.7%, $P \leq .05$) were closer to the values for red-orange heads obtained by the TAO method with AO. There was no statistical difference among absolute values for the TB, AB, and TAO methods, whereas

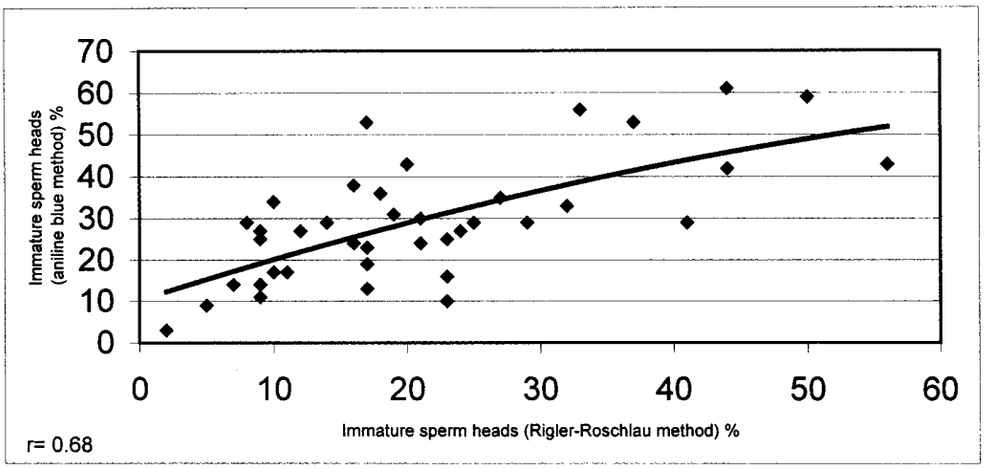


Figure 4. Relationship between the percentage of immature sperm cells revealed by the RRAO and AB methods.

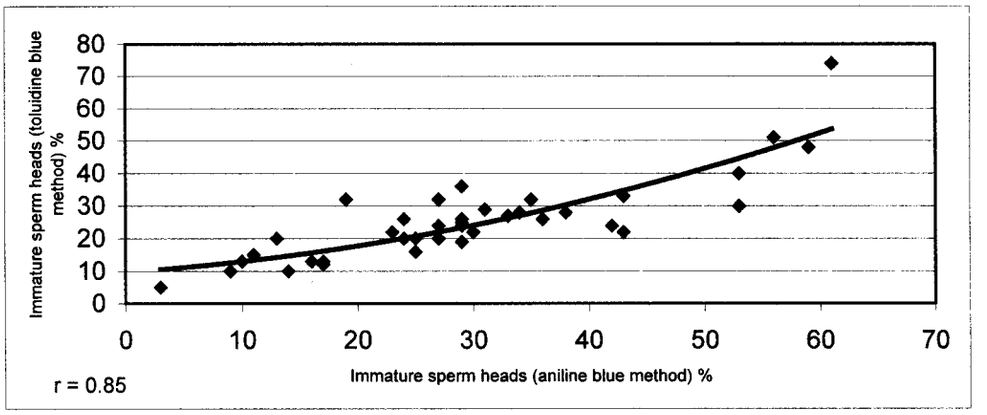


Figure 5. Relationship between the percentage of immature sperm cells revealed by the AB and TB methods.

absolute values for the red-orange heads obtained by the RRAO method were credibly lower.

Morphological examination of spermatozoa stained with TB showed that deep violet sperm heads were more often larger and of pathological forms than the blue ones. However, we also found violet sperm heads of quite normal size and morphology.

It has been reported in the literature that the difference in the proportion of sperm cells with abnormal, easily denaturable DNA is not statistically significant between infertile and fertile men (Tejada et al, 1984; Hoshi et al, 1996). However, when the count of cells with normal DNA is multiplied by the sperm concentration per milliliter, obtaining the ''effective sperm count,'' the fertile and infertile groups can be easily separated. Fifty million and above was a borderline of sure fertility found with the TAO method, and our data applying this approach using the modified RRAO method on 60 semen samples gave similar results.

In our study, detection of the fertility threshold comparing the average values of cells with impaired DNA integrity using the RRAO method were 17% ± 3.8% and 30.2% ± 6.8%, $P \leq .05$, for the fertile and infertile groups, respectively, setting a threshold of 95% probability at 23%. The average values, using the TAO method, were 23.9% ± 7.5% and 52.1% ± 20.8%, $P \leq .05$, for the fertile and infertile groups, respectively, setting a threshold at 31% (Table).

## *Discussion*

This study characterizes all 4 is situ techniques used as adequate to estimate in situ sperm DNA integrity and chromatin structure, proving the expedience of the recently introduced RRAO and TB methods alongside the well-known, widely used AB and TAO methods. The slight differences are most probably due to the different

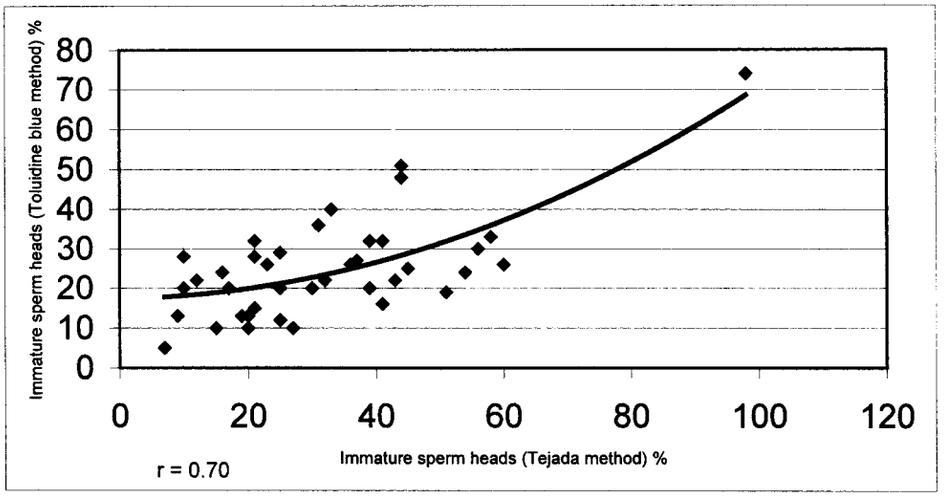


Figure 6. Relationship between the percentage of immature sperm cells revealed by the TAO and TB methods in 40 human ejaculate samples.

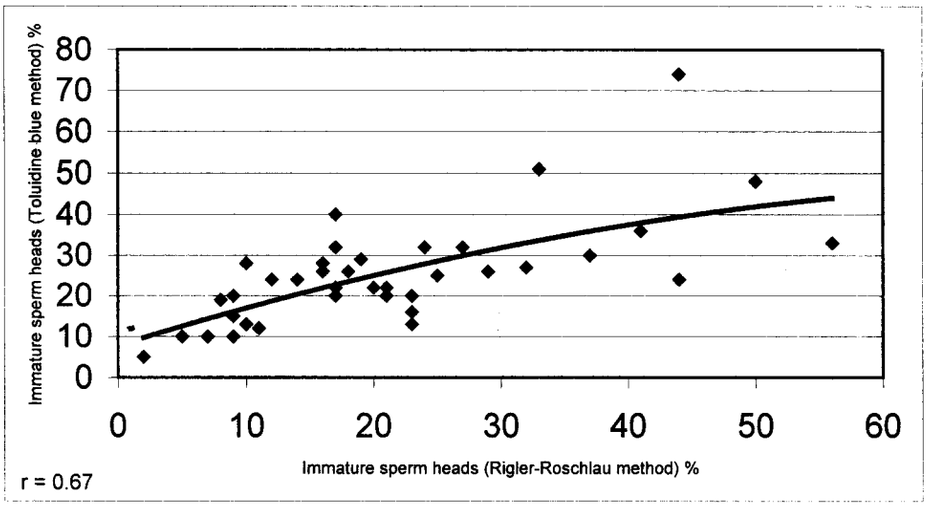


Figure 7. Correlation between the percentage of immature sperm cells revealed by the RRAO and TB methods in 40 human ejaculate samples.

fixation methods. The closest averages were between the RRAO and TB methods using the same fixation, which has been recommended by Rigler (1966) as the best preserving natural relationship between nucleic acids and associated proteins.

A fertility threshold level was recently determined by Evenson and colleagues (1999). The difference in proportion of sperm cells with abnormal, easily denaturable DNA stained by AO and evaluated by the SCSA method between infertile and fertile men found in this study suggests a threshold at 30%, which is possibly compatible with fertility. The values obtained by us in the present study are close to that value.

The phenomenon of fertility threshold and of effective sperm count is interesting; in fact, fertilization is performed by one single spermatozoon. However, one can calculate that in most cases, excluding full azoospermia, there should be still more than enough cells reaching the oocyte with normal-staining DNA to provide natural penetration. Therefore, it seems that differentiation of good and bad sperm cells (ie, containing normal and abnormal DNA, respectively) as red and green in the AO test or violet and blue in the TB test, is to some extent arbitrary. It can be hypothesized that due to the cooperative (cascade-like) nature of metachromatic staining (Goldstein, 1963; Sculthore, 1978), which in these tests is indicative of bad sperm chromatin integrity, only severe DNA damage is revealed. Less severe damage remains under the threshold of the cooperative metachromatic effect. This can be inferred from the presence of light violet sperm heads in parallel to light blue and deep violet sperm heads in our material, as was also indicated by Andreetta et al (1995) in TB-stained samples and these should represent an intermediate group, between arbitrary normal and abnormal, revealed by TB. Further elaboration of the methods to discriminate this group, which requires automated spectrophotometry, can increase the validity of such a probe in predicting fertilization prognosis.

Our method of TB staining and that of Andreetta et al (1995) have the same cytochemical principle and, although different in their methodical details, should provide similar results; however, we have not examined this. The finding that violet sperm heads can also appear to have normal morphology and size is in line with previous data using the AO and AB tests, which show that sperm heads with impaired chromatin structure frequently have pathological morphology but may also have normal morphology (Dadoune et al, 1988; Liu and Baker, 1992). Therefore, the correlation between red cell counts and pathological forms was not very high in our material ($r = .38$, $P < .01$), and is similar to the data of other authors (Tejada et al, 1984; Liu and Baker, 1994; Sukcharoen, 1995).

Because red fluorescence by AO sperm heads was shown to have damaged DNA, by correlation with in situ detection of the DNA strand break-containing cells (Evenson et al, 1986; Evenson, 1990; Gorzyca et al, 1993), by implication, deep violet sperm heads must also belong to this class.

Tejada's method is less labor-consuming and a little more sensitive to damaged DNA compared with the modified Rigler-Roschlau method, whereas in the latter method, AO does not fade so dramatically and the slides can be evaluated during the next 48 hours. The TB and AB methods are simple and inexpensive and have the advantage of providing permanent preparations for use on an ordinary light microscope. The smears stained with the TB method can be also used for morphological estimation of cells. For this purpose, TB has the advantage in comparison with the AB method because it is difficult to as-

sess the morphology of unstained sperm heads by the latter method, which would appear blue using the TB method.

The methods on slides have the inherent limits of repeatability dictated by dye equilibrium variations and by a limited number of cells, which can be reasonably scored. In contrast, the flow cytometry SCSA test is free of these problems. However, given the modest number of semen samples studied in this research and the closeness of the obtained parameters to those reported by the SCSA method on a large material (Evenson et al, 1999), these methods seem to be good enough to consider them for practical use.

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## *Appendix: Mechanisms of the Chromatin Structural Probes*

Several factors influence staining of the chromatin by planar ionic dyes: 1) the secondary structure of DNA, 2) regularity and density of the chromatin packaging, and 3) binding of DNA to chromatin proteins. In addition, dye molecule structure and size, charge, concentration, pH, molarity, and staining mechanism (intercalary, external, or both) play important roles. In the intercalary mechanism, the planar dye molecules are inserted between the DNA nitrogen bases, but not in every pair, and therefore, they cannot undergo polymerization. However, with the action of an external mechanism, the dye molecules become attached to the phosphate groups of the DNA sugar-phosphate backbone and, subsequently, are close enough to interact and polymerize on the substrate.

*DNA Secondary Structure and Conformation*—Fragmented DNA is easily denaturable (Darzynkiewicz et al, 1975). However, even a single DNA strand break causes conformational transition of the DNA domain from the superspiralized state to the relaxed state. Superspiralized DNA avidly takes up intercalating dyes (providing green fluorescence by AO) because this reduces the free energy of torsion stress. By contrast, the affinity for the intercalation is low in the relaxed DNA and is lost in single-stranded DNA. In this case, an external mechanism of dye binding to DNA phosphate residues (providing the enhanced red DNA fluorescence by AO) is favored (Erenpreisa et al, 1988a, 1988b). Nevertheless, nicking or fragmentation of DNA is not the only factor in the competition between metachromatic versus orthochromatic staining. Their proportions are also influenced by the chromatin packaging density (see later discussion). In addition, chromatin proteins affect external DNA dye binding because they are differently bound to the relaxed or superspiralized DNA (see later discussion). Moreover, the presence of disulfide bonds in the chromatin of mature sperm can prevent denaturation of DNA (Evenson et al, 1986).

Of particular interest may be the conformational changes of the chromatin in round spermatids. It appears that initial displacement of histones from DNA should cause the torsion stress in DNA domains, which may be relaxed by introduction of single-strand breaks. DNA relaxation would favor the cooperative release of the nucleosome cores from the DNA (Benyajati and Worcel, 1976). The relaxed DNA then can easily interact with transient proteins and protamines to provide the very tight packaging of sperm chromatin. It can be suggested that this process may occur gradually during maturation along the axis of spermatid in one DNA domain after another. This could explain the anomalous staining properties of round spermatids found by Evenson et al (1986), indicating a non-stoichiometric increase of nuclear staining by nearly all nuclear dyes, with both intercalative and external mechanisms. It can also explain our finding of metachromatic staining of round spermatids and bicolored polar staining of caudal spermatozoa in rat spermatogenesis.

*Chromatin Packaging Density*—If the chromatin is regularly arranged and sufficiently densely packed, dye polymerization providing metachromasy (color change) is favored (Sculthore 1978; Erenpreisa and Zaleskaja, 1983). If the chromatin is very loosely packed, or the dye is very diluted, or both, only monomolecular orthochromatic staining is possible. However, if the chromatin is packed even more densely (as in normal sperm), the polymerization of the dye can be hindered again, which favors a shift to orthochromatic (chaotic) staining (Erenpreisa et al, 1992) and may even prevent dye binding, especially by large, bulky dyes at an unfavorable pH. The latter occurs when AB is used, an acid dye that stains basic proteins associated with DNA at low pH.

*Chromatin Proteins*—Superspiralized DNA requires covalent binding of some nuclear matrix proteins and tight ionic interaction between DNA and chromatin proteins to support negative supercoils (Benyajati and Worcel, 1976). Relaxed DNA has a looser ionic interaction with chromatin proteins, which can be more easily displaced from the DNA, thus favoring the cooperative, external metachromatic binding of the dye to DNA phosphate groups. On somatic thymocytes, acid pretreatment with the AO method can be replaced by 0.4 M NaCl, which removes loosely bound proteins to ensure metachromatic staining of the damaged DNA (eg, in early apoptotic cells; Erenpreisa et al, 1997). This method does not work on sperm heads on which disulfide bonds restrict ionic displacement of proteins; however, their reduction by dithiothreitol gives an effect that can replace acid pretreatment (reported by Andreetta et al, 1995).

Thus, chromatin structural probes show a net cooperative contribution by the DNA secondary structure and conformation, the chromatin packaging density, and the binding strength and content of the proteins interacting with DNA.