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Effect of Deoxyribonucleic Acid Protamination on Fluorochrome Staining and In Situ Nick-Translation of Murine and Human Mature Spermatozoa[1]

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ABSTRACT

A major event in enhancing sperm chromatin stability is the replacement of the histones by protamines during spermiogenesis. In this study, we present results indicating that chromomycin A3 (CMA_3) can be used to show protamine deficiency in sperm chromatin. Fixed chromatin of mature mouse spermatozoa showed high fluorescence after treatment with ethidium bromide (EB), but was completely unstained after treatment with CMA_3. The same chromatin was found to be highly resistant to in situ nick-translation. In contrast, a substantial fraction of human spermatozoa were positive for CMA_3. The accessibility of CMA_3 to the DNA of human sperm was eliminated if the slides were previously treated with protamine in situ. This treatment did not affect the accessibility of EB to the chromatin. Individual human sperm samples revealed a substantial frequency of spermatozoa with endogenous nicks, which was found to be the same as the frequency of spermatozoa responding positively to CMA_3 staining. Treatment of preparations with protamines prevented the identification of the endogenous nicks. These data as a whole suggest that CMA_3 could represent a useful tool for the detection of protamine deficiency in sperm chromatin. Furthermore, confirmation of experiments relating sensitivity to nick translation and positivity to CMA_3 may allow an indirect in situ visualization of nicked and partially denatured DNA, which could correlate with certain forms of male factor infertility.

INTRODUCTION

The chromatin contained in the nuclei of mature mammalian spermatozoa is an extremely compact and stable structure. Its very high packaging quality is principally due to dramatic modifications of the nucleoprotein components, the most essential consisting of the replacement of histones by protamines, occurring during spermiogenesis [1–9]. A model of protamine-DNA binding holds that protamines bind to a nucleic acid strand by lying lengthwise inside its minor groove and then fitting into the major groove of a neighboring strand. In this way, different segments of DNA may be arranged side by side to form a grid of tightly packaged non-supercoiled DNA [6, 9]. Further stabilization is then accomplished by the formation of intra- and intermolecular disulphide cross-links among the cysteine residues of the protamine molecule. The structure of this insoluble, inactive chromatin is therefore very different from that found in the genetically active somatic nucleus. In fact, after release from the testis, the sperm chromatin is not only refractory to in situ enzymatic treatment [10, 11], but also reveals a progressive decrease in Feulgen stainability, as well as a variable reduction in accessibility and binding capacity to many dyes and fluorochromes [12–18]. In the past decade, the variations in accessibility to DNA have been analyzed in order to establish hypothetical correlations between sperm nuclear instability and conditions of subfertility and infertility in mammals, including man [11, 19–22]. In one such study, Monaco and Rasch [23], using DNA fluorochromes specific for GC-rich sequences, like mithramycin or chromomycin A3 (CMA_3) and for AT-rich sequences like Hoechst 33258 or 4–6, diamidino-2-phenylindole (DAPI), found brilliant chromatin fluorescence with both types of fluorochromes in primary and secondary spermatocytes and in young spermatids. The authors concluded that the decline in staining with GC-specific dyes observed in maturing spermatozoa of vertebrates probably reflected changes in protein composition and in DNA packaging ratios that restricted the availability of previously reactive sites.

Following this line of research, we present data, obtained by studying both murine and human mature spermatozoa, suggesting that 1) the amount of protamines present in mature sperm seems to represent at least one of the limiting factors controlling the accessibility of CMA_3 to the DNA; 2) protamines also protect the tightly packaged DNA of the sperm head from attack by DNA-polymerase I and thus from in situ nick-translation; and 3) CMA_3 could be useful as a tool for use in routine laboratory analysis for the rapid screening of certain conditions of subfertility and infertility in man, as it seems to allow an indirect visualization of protamine-deficient, nicked, and partially denatured DNA.

MATERIALS AND METHODS

Sperm Preparation

Twelve- to sixteen-week-old OF1 mice were obtained from BRL (Basel, Switzerland). Sperm cells were collected by

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gently squeezing the vas deferens and cauda epididymis, with two fine forceps. The sperm were then incubated in medium M16 [24] supplemented with 15 mg/ml BSA (type V; Sigma, Buchs, Switzerland) for 1 h, to allow for cell dispersion. The sperm were then collected, washed twice in PBS by centrifuging at 1300 rpm, fixed in methanol/acetic acid, 3:1, for 5 min, spread on slides, and air-dried. Ejaculated human spermatozoa, from both fertile and infertile men, were washed in Dulbecco's Ca^{2+}-Mg^{2+}-free PBS, (two parts PBS and one part ejaculate) and centrifuged at 1200 rpm for 10 min. The procedure was repeated twice, and the washed spermatozoa were fixed in methanol/acetic acid, 3:1, at 4°C, for 5 min and then were spread on slides.

Ethidium Bromide Staining, Chromomycin Staining, and In Situ Protamine Treatment

For ethidium bromide (EB) (Sigma) staining, slides were treated for 30 min with a 0.04% solution of EB in PBS, pH 7.4. Slides were then rinsed in distilled water, air dried, and mounted in PBS buffer.

For CMA_3 staining, each slide was treated for 20 min with 100 μl of CMA_3 solution (0.25 mg/ml in McIlvaine buffer, pH 7.0, containing 10 mM $MgCl_2$). Slides were then rinsed in buffer, air-dried, and mounted with a mixture of buffered glycerol and *p*-phenylendiamine [25].

In situ protamination of murine and human spermatozoa was carried out by treating each fixed preparation for 30 min with 100 μl distilled water containing 5 mg salmon protamine (Sigma).

In Situ Nick-Translation

For in situ nick-translation the fixed preparations were preincubated with 50 ng/ml DNase I in DNase buffer (1 mg/ml BSA in 10 mM Tris-HCl, pH 7.5) for 30 min or, alternatively, with 200 U/ml of the restriction endonuclease Alu I in the appropriate buffer recommended by Boehringer (Milan, Italy) for 1 h. A number of experiments on human sperm chromatin were performed by omitting the above-mentioned endonuclease treatments, since in the presence of preexisting DNA endogenous nicks, the DNA polymerase I, by virtue of its 5′-3′ exonucleolytic activity, can catalyze movement of the nicks along the double helix [26]. The nick-translating procedure was performed according to Sumner et al. [27], except that streptavidin fluorescein isothiocyanate (FITC) was used as a label instead of streptavidin alkaline phosphatase to evidence the incorporation of biotinylated d-uridine triphosphate (d-UTP). The fluorescent staining solution was prepared by mixing 10 μl SAHR FITC, 90 μl Tris buffer, pH 7.5, and 900 μl bidistilled water. One hundred microliters of this diluted solution was added to each slide and covered by a coverslip. The incubation was carried out in a moist chamber at 37°C for 30 min. After incubation, slides were rinsed twice in PBS, washed in distilled water, and finally mounted with a mixture of buffered glycerol and *p*-phenylenediamine. Fluorescence studies were performed with a Zeiss Photomikroskop III equipped with a photometer 03 microfluorimeter. The combination of exciter: dichroic: barrier filters were BP 546/12: FT 580: LP 590 for EB; BP 485: FT 510: LP 520 for FITC, and BP 436/10: FT 460: LP 470 for CMA_3.

RESULTS

Murine Spermatozoa

All mature spermatozoa obtained from mouse cauda epididymis and vas deferens showed high fluorescence after treatment with EB (Fig. 1a). In contrast, when CMA_3 instead of EB was used, nearly all the spermatozoa treated with CMA_3 were nonfluorescent, with very few exceptions, since fewer than 1% of the spermatozoa were CMA_3 positive (Fig. 1c). In situ protamination of the slides did not change the general picture described above. In fact, preparations pretreated with salmon protamines reacted positively with EB (Fig. 1b), showing a fluorescent emission that, when estimated microfluorimetrically, was practically unchanged from that of the untreated controls (180.6 ± 11 arbitrary units [a.u.], in untreated and 182.2 ± 12 in protaminated sperm, respectively). Slides of protaminated mouse sperm were also CMA_3-negative (Fig. 1d).

The fixed chromatin of mature mouse spermatozoa was found to be highly resistant to in situ nick-translation: almost none of the spermatozoa were found to incorporate biotinylated d-UTP, in the presence of DNA-polymerase I, after treatment with either DNase I or Alu I.

Human Spermatozoa

A substantially different situation was observed in ejaculated human spermatozoa. These cells were in fact all EB-positive (Fig. 2a), but unlike the mouse cells, a number of them were also positive when treated with CMA_3. The percentage of CMA_3-positive human sperm varied greatly between individual donors, being roughly under 15%–20% in normospermic donors, but constantly showing higher values in the infertile-male-factor donors. In one sample, the percentage of CMA_3-stained spermatozoa reached the unusual value of 70% (Fig. 2c). The unexpected high accessibility of CMA_3 to the DNA of several donors' sperm was completely eliminated when the slides had previously been treated with salmon protamines, both in spermatozoa obtained from normospermic and in those from infertile-male-factor donors (Fig. 2d). As in the mouse, the in situ treatment of human spermatozoa with protamines apparently did not affect the accessibility of EB to the chromatin (Fig. 2b). In fact, we found microfluorometrically the same values of emission both in untreated controls and in protaminated slides (221.9 ± 10 a.u. and 218.6 ± 12 a.u., respectively). In contrast to murine spermatozoa, human preparations revealed a substantial amount of nick-trans-

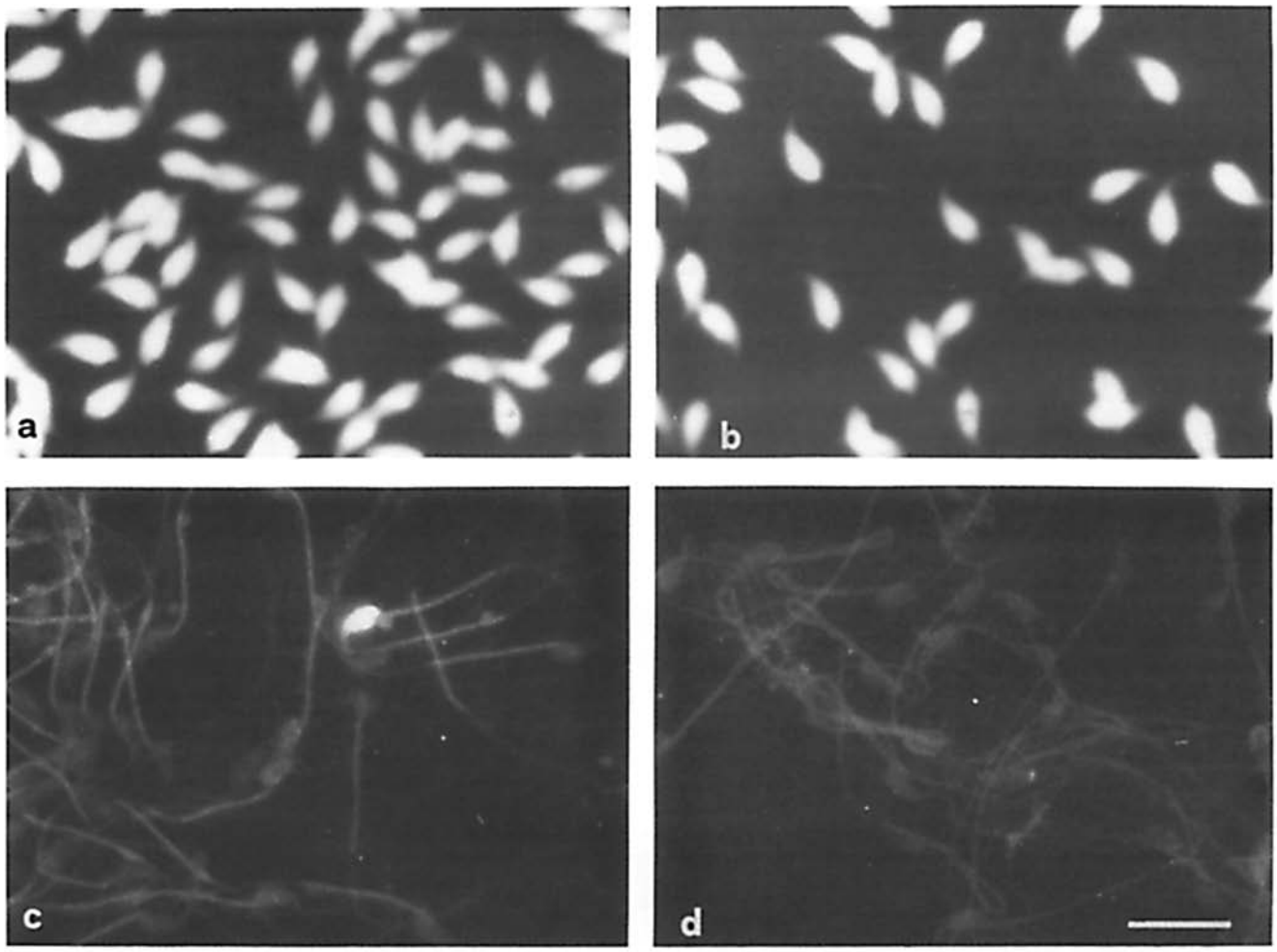

FIG. 1. Mature murine spermatozoa stained with ethidium bromide (a) before and (b) after treatment with salmon protamines and CMA_3 (c) before and (d) after treatment with salmon protamines. Bar = 10 μm.

lated spermatozoa (Fig. 2e). In respect to this, three interesting observations must be emphasized: 1) Incorporation of biotinylated d-UTP in sperm chromatin was also obtained by simply incubating the slide with DNA-polymerase I and omitting digestion with both aspecific and specific endonucleases, revealing the presence of endogenous nicks. This result, interestingly, was not observed in diploid somatic cells that happened to be present on the same slide. 2) In individual specimens, the frequency of sperm responding positively to the nick-translating action of DNA-polymerase I was found to be constantly similar to the frequency of sperm that responded positively to staining with CMA_3. 3) As in the case of the eliminated CMA_3 positivity referred to above, the treatment of the slides with salmon protamines also prevented in situ nick-translation from taking place (Fig. 2f).

DISCUSSION

Chromatin of mature mammalian spermatozoa has repeatedly been shown to possess a low binding capacity for several dyes and fluorochromes, such as methyl green, Giemsa stain, ethidium bromide, propidium iodide, acridine orange, acriflavin, DAPI, mithramycin, and toluidine blue [17, 18, 22, 28–32]. It has been suggested, and is generally accepted, that the compact, insoluble structure of the sperm chromatin limits and in some cases prevents the access to DNA of intercalators, minor groove ligands, and phosphate-binding dyes. Therefore, an important factor responsible for the low staining of mature sperm chromatin could be the complete neutralization of DNA phosphates by protamines together with the occurrence of interprotamine cross-links [6, 7, 33].

As previously stated, Monaco and Rasch [23] suggested that the decline in mithramycin and CMA_3 staining intensity, observed in maturing sperm of fish, frogs, and rabbits, probably reflects changes in protein composition and in DNA packaging ratios. This suggestion received circumstantial support from an experiment by Evenson et al. [34], who analyzed in mice the effect of HCl-extractable chromatin constituent on DNA accessibility to several fluorochromes,

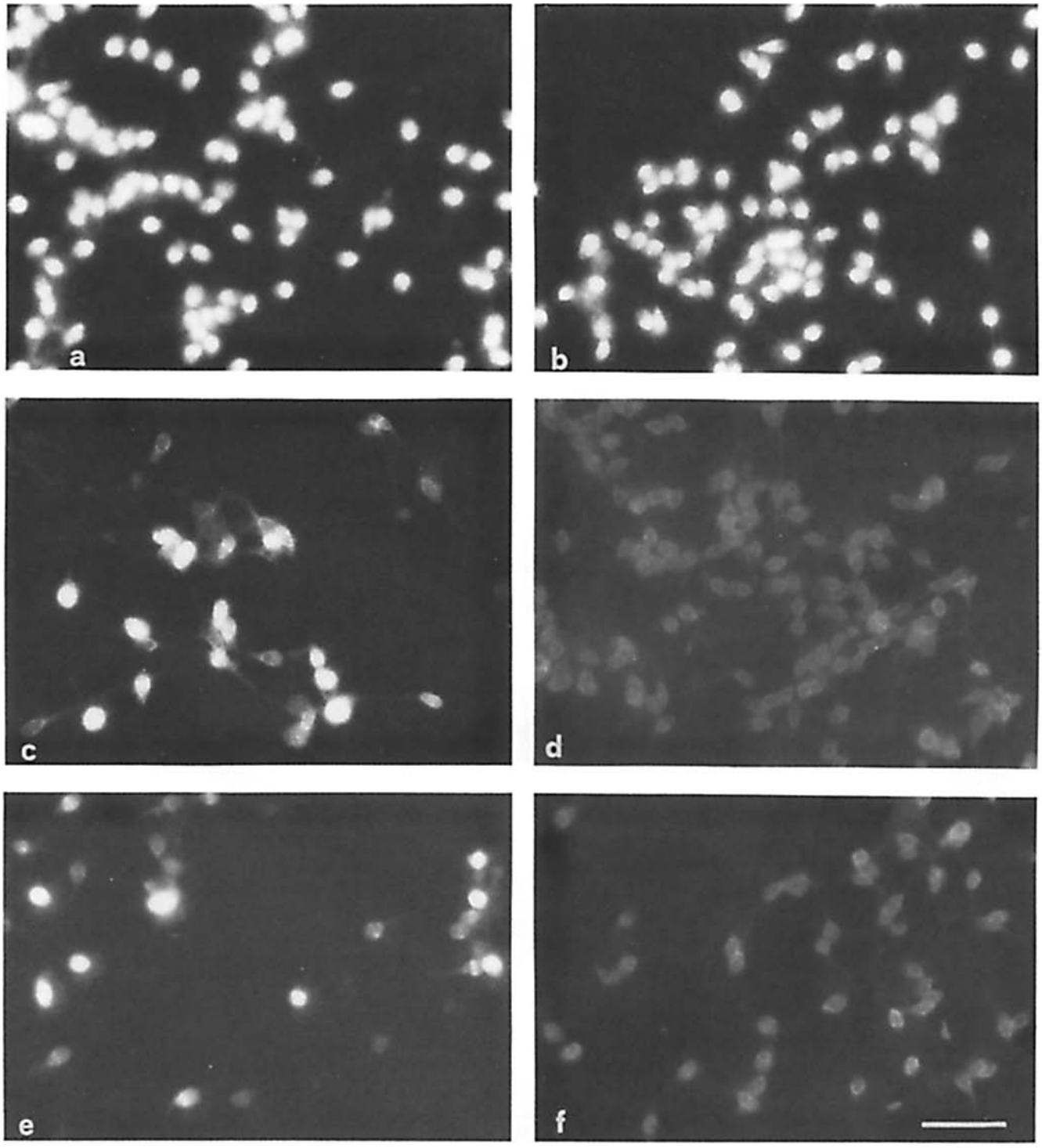

FIG. 2. Ejaculated human spermatozoa stained with ethidium bromide (a) before and (b) after treatment with salmon protamines; CMA_3 (c) before and (d) after treatment with salmon protamines; and FITC after nick-translation (identifying endogenous nicks in the sperm chromatin) (e) before and (f) after treatment with salmon protamines. Bar = 10 μm.

at various stages of sperm differentiation. These authors observed that HCl treatment of vas deferens sperm increased the staining level of both intercalating and externally binding dyes, including CMA_3. They also noted that elongated spermatids, which contain a mixture of protein types including histones, transition proteins, and protamines, showed the greatest variability of staining with respect to the type of stain and effect of acid extraction of proteins.

The differential accessibility of CMA_3 to sperm chromatin that our experiments have shown both between species (mice and humans) and within species (humans) emphasizes the important role protamines play in both tightly packaging and protecting the haploid genetic project of mature spermatozoa. Different models have been proposed for the binding of protamines to DNA in spermatozoa [6, 33, 35]. These models postulate that the polyarginine segment of protamine binds in the minor groove of DNA, cross-linking and neutralizing the phosphodiester backbone. The precise mechanism of binding of CMA_3 to the double helix has been the subject of a number of studies; however, it remains uncertain [36–39]. For instance, it is still unclear whether the chromomycinone ring of CMA_3 can also bind the double helix via an intercalative mechanism, even though it is generally accepted that the large and relatively lipophilic didedoxy-sugar side chains of CMA_3 may be located preferentially in the minor groove of B-DNA, due to hydrophobic interactions. It is therefore feasible to hypothesize the existence of a molecular competition between CMA_3 and protamines for interaction with the minor groove of DNA. The fact that salmon protamines, utilized in situ, were able to preclude CMA_3 accessibility to DNA of originally CMA_3-positive human sperm, strongly supports this suggestion, especially considering the lack of accessibility of the same fluorochrome to the normal and naturally protaminated murine chromatin. On the other hand, artificially protaminated human chromatin was completely dark only after treatment with a ligand such as CMA_3, while the fluorescence emitted by in situ-protaminated sperm heads stained with a classic intercalating dye like EB provided no quantitatively measurable difference in either murine and human spermatozoa. In relation to this, we stress that although it is well known that protamines of fish and mammals differ both in size and amino acid sequences, it is generally assumed that the mechanism of chromatin organization in the spermatozoa of these organisms is similar [6].

As we have already stated, nick-translation experiments performed on human preparations constantly showed an appreciable uptake of biotinylated d-UTP, even when the slides were simply incubated with DNA-polymerase I. The frequency of spermatozoa revealing endogenous nicks in their chromatin was constantly similar to the frequency of CMA_3-positive spermatozoa. Moreover, the treatment of these slides with salmon protamines hindered nick-translation. Certain inferences can be made from this experiment. First, it seems possible that mature human sperm containing underprotamined DNA regions, which stain with CMA_3, can also be naturally nicked and/or partially denatured, thus increasing their susceptibility to enzymes having 5′-3′ exonuclease activity, such as DNA-polymerase I. The naturally highly protaminated chromatin of murine spermatozoa, was resistant to both nick-translation and CMA_3 staining, behaving exactly like the artificially protaminated human sperm chromatin. These findings partially fit the data of Adolph et al. [10], who showed that sperm cells were highly sensitive to DNase I during the later stages of spermatogenesis, but resistant as mature spermatozoa. Second, murine and human diploid cells, which possess a nucleosomal organization of their chromatin and were present on the artificially protaminated slides, responded positively to CMA_3 staining and negatively to the 5′-3′ exonuclease activity of DNA-polymerase I. This observation suggests that salmon protamines interact in vitro only with sperm chromatin. Finally, our experiments suggest that protamines can protect sperm DNA against the enzymatic attack of nucleases and polymerases even in situ. Interestingly, it is noteworthy to point out that CMA_3 has also been experimentally found to protect DNA against the action of nucleases and polymerases [36]. The use of CMA_3 in routine screening of human spermatozoa could therefore represent a useful tool for the detection of protamine deficiency in sperm chromatin. Correspondingly, only additional studies will show if CMA_3 positivity also represents an indirect visualization of nicked and damaged DNA.

The results from this study and from other laboratories [35, 37, 38] strongly suggest that both CMA_3 and protamines may bind through the minor groove of DNA and thus compete for the same site, even if the mode of binding may be different. Most importantly, our experiments have shown that the accessibility of CMA_3 to spermatozoa from infertile-male-factor patients can be altered by protamination. A correlation of CMA_3 positivity and male infertility would imply that certain male-factor patients show inherent anomalies in spermiogenesis during the replacement of histones by protamines. This in turn would imply that spermatozoa are rendered functionally immature by lack of protamination and that anomalies in chromatin structure may limit the fertilizing ability of spermatozoa.

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