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EXTENDED CHROMATIN FIBERS IN MOUSE TESTICULAR SPERMATOZOA*

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ABSTRACT

Since in mouse spermatozoa the somatic histones are replaced by other basic proteins and there are changes in the chromatin supraorganization, different patterns of extended chromatin fiber (ECF) formation would be expected compared with those formed by somatic cells that were previously studied. In this study, we investigated the formation of ECF in mouse testicular spermatozoa after lysis with 2 M NaCl plus 1% Triton X-100, and under the action of gravity. ECFs were observed under polarized light in fixed and unfixed spermatozoa subjected to lysis in a vertical position and stained with toluidine blue at pH 4.0. In unfixed preparations, all of the sperm nuclei showed ECFs, whereas in fixed preparations 60% of the cells had ECF. The latter frequency was much higher than that previously reported for mouse hepatocytes. Even in cells that did not produce ECFs in vertically and horizontally lysed preparations, an ordered reorganization of the chromatin was observed after lysis. The faint positive response to acid fast green at the nuclear periphery in spermatozoa that did not develop ECF after lysis was assumed to represent residual protamine and nuclear matrix proteins. The high frequency of mouse sperm cell nuclei with ECF probably reflected the extraction of protamines from the DNA-protein complexes of sperm cell nuclei facilitated by the specific lysis protocol.

Key words: Chromatin extensibility, mouse, optical anisotropy, testicular spermatozoa, topochemistry

INTRODUCTION

Chromatin and DNA flow from cell nuclei following treatment with lysis solutions that remove RNA, histones, and some of the non-histone proteins from chromatin, as well as part of the lipids from the nuclear envelope. As a result, extended chromatin fibers (ECF) can be formed under the action of gravity or during manual mechanical stretching [5-7,14,21]. ECF formation depends on the rheological properties of the DNA [13,18] and also on proteins that contribute to higher-order chromatin structure, as in the case of nuclear matrix proteins [11,17,19,20,22]. Chromatin extensibility under gravity in adult mouse hepatocyte nuclei is affected by the organism's nutritional state, such that the frequency of nuclei that form ECF under the same treatment conditions decreases drastically following starvation and is accompanied by an increase in chromatin condensation [11].

In sperm cells, drastic changes in chromatin composition compared to somatic cells have been reported, with somatic histones being totally or partially replaced by histone-like basic proteins [1,12]. In spermatozoa-containing protamines (arginine-rich; no lysine), protamine-like histones (arginine-rich, little or no lysine, but oxidized cysteine also being present), or intermediate basic sperm proteins (containing histidine and/or lysine in addition to arginine), these proteins tightly pack the DNA in most animal species, whereas in spermatozoa-containing somatic-like lysine-rich histones, such as honey bees, sea-urchins and *Rana*, the DNA-protein complex is much less tightly packed [1,4,9,12]. Protamine-like histones are usually found in eutherian mammals, with mice having two protamine-like variants [2].

The supraorganization of chromatin in mammalian sperm cells depends on the basic nuclear protein types and on a nuclear matrix. Sperm cell DNA organizes in loop domains that are attached to the nuclear matrix by their matrix attachment regions (MARs) [22]. Since the DNA in sperm cells is not involved in transcriptional processes, the regions of DNA attachment to the nuclear matrix probably has only structural functions [16].

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*Dedicated to Professor Benedicto de Campos Vidal on the occasion of his 75th birthday.

In addition to the extremely compact structure of chromatin in mammalian sperm, different hierarchies and heterogeneity exist in the packaging of DNA in the nucleus of these cells [6]. Hence, the DNA-protein complexes of mammalian sperm cells are expected to behave differently from those of somatic cells in the same species, and this behavior is likely to be influenced by the nutritional state and developmental stage, as well as by treatment with high salt and detergent solutions that destabilize these complexes.

In the present study, the formation of ECF was investigated in mouse testicular spermatozoa and the findings were compared with data for mouse hepatocytes [11] under the same treatment conditions.

MATERIAL AND METHODS

Male mice of the inbred strain A/Uni, obtained from the Multidisciplinary Center for Biological Investigation of the State University of Campinas (CEMIB/UNICAMP) were reared under normal conditions and fed standard extruded chow (Purina®) *ad libitum* until 15 weeks old. Four specimens were killed by decapitation and their testes were immediately removed, placed in cold saline solution (0.9% NaCl in distilled water), and used for the preparation of imprints on glass slides. All of the protocols involving animal care and use were approved by the Committee for Ethics in Animal Experimentation of the State University of Campinas (CEEA/IB) and met the guidelines of the Canadian Council on Animal Care.

Treatments

Freshly prepared imprints were fixed in absolute ethanol-glacial acetic acid (3:1, v/v) for 1 min and then rinsed in 70% ethanol for 5 min. The slides were positioned vertically and horizontally and the imprints were immediately lysed in 2 M NaCl plus 1% Triton X-100 in Tris-HCl buffer (25 mM, pH 7.4) for 5 h at 25°C to obtain ECFs, after which the volume of solution was completed with absolute ethanol to a final concentration of 50% for 10 min. Following this, the slides were removed from the lysis solution and transferred to 70% ethanol for 30 min [5,7,21]. Unfixed preparations subjected to the same lysis protocol but incubated for a shorter period of time (10 min) [11] were also used. Fixed preparations that had not been subjected to the lysis protocol were used as treatment controls.

Topochemistry and optical anisotropy

Staining was done with a 0.025% toluidine blue (Merck, Darmstadt, Germany) solution in McIlvaine buffer at pH 4.0 for 15 min [20,21]. The preparations were then rapidly (5 s) rinsed in distilled water, air dried, cleared in xylene, and mounted in natural Canada balsam. This staining procedure was used because: 1) it is a classic method in cytochemical and biochemical assay of nucleic acids; 2) the molecules of this dye bind electrostatically to available DNA and RNA phosphates; 3) it is possible to detect the optical anisotropic characteristics (birefringence and linear dichroism) of stained DNA or DNA-protein complexes, inclusive in ECFs [11,19,21], under suitable conditions [8,10,20]. Birefringence and linear dichroism (selective absorption of polarized light) in toluidine blue-stained chromatin reveals the availability and proximity of free DNA phosphates suitable for dye binding and the oriented arrangement of the dye binding sites in chromatin [8,10,19]. The frequency of nuclei showing ECFs was estimated under polarized light. Birefringence was investigated in toluidine blue-stained cells under polarized light using a Zeiss polarizing microscope equipped with a 16/0.32 Planachromatic objective, and was photographed in a Zeiss Axiophot 2 microscope equipped with 40/0.75 and 100/1.30 Pol-Neofluar objectives, optovar 2, and a 1.4 condenser. Kodak Gold 100 film was used for the photomicrographs.

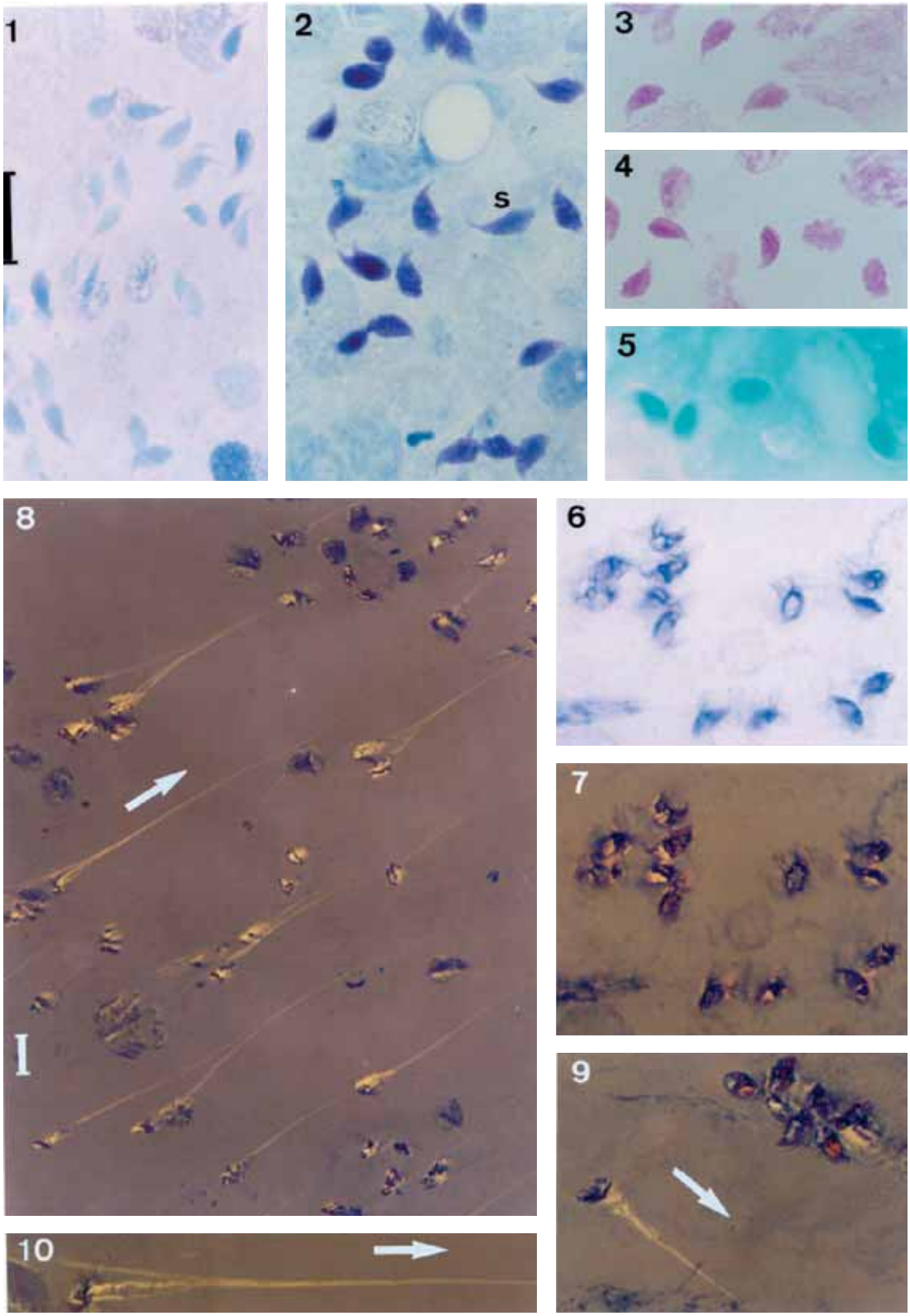
Some preparations were also subjected to the Feulgen reaction, a highly specific, stoichiometric reaction to detect DNA. Acid hydrolysis pertinent to this assay was done in 4 M HCl at 25°C for 60 min. Some preparations were also screened for the presence of total nuclear proteins using acid fast green (Sigma, St. Louis, USA), in which the anionic dye binds electrostatically to the available cationic groups of proteins [3,8].

RESULTS

Sperm cell nuclei that were not subjected to lysis stained light blue (unfixed preparations) or deep violet (fixed preparations) with toluidine blue (Figs. 1 and 2). The sperm cell nuclei also stained intensely with the Feulgen reaction and by the acid fast green method (Figs. 3-5). The toluidine blue-stained spermatozoal nuclei showed no birefringence when examined with a crossed analyzer and polarizer.

When the fixed preparations were subjected to lysis horizontally or vertically and then stained with toluidine blue, weak metachromatic staining was seen in the center of the sperm cell nuclei, and was surrounded by stronger staining at the nuclear periphery; a deeply birefringent nuclear image was also observed (Figs. 6-8). In slides positioned vertically, toluidine blue-stained filaments were seen flowing from some nuclei in the form of ECFs (Figs. 8-10). Approximately 60 % of the sperm cells showed ECFs but no ECFs were seen in cells other than spermatozoa (data not shown).

The ECFs were stained in the Feulgen reaction but not by the acid fast green method (Fig. 11). Fixed



Figures 1-5. Unfixed (**1, 3**) and fixed (**2, 4, 5**) mouse testicular imprints (control) stained with toluidine blue (**1, 2**), the Feulgen reaction (**3, 4**) and acid fast green (**5**). **s**, spermatozoa.
Figures 6-10. Fixed and vertically lysed mouse sperm cells treated with toluidine blue. **7** is a polarized light view of **6**, showing spermatozoa that did not form prominent ECFs. **8-10**. General view (**8**) and details (**9, 10**) of ECFs as seen under polarized light. The arrows indicate the direction of gravity. Bars = 20 μm.

sperm cells that were subjected to lysis showed nuclear images that stained faintly with acid fast green, whereas the sperm cell tails were deeply stained (Fig. 12). Compared to the sperm cells, the nuclei of the spermatogonia showed filamentous, granular elements and a nucleolar-like body that stained very well with acid fast green (Fig. 13).

In unfixed preparations treated vertically with the short lysis protocol, practically all of the sperm cell nuclei produced long ECFs that stained metachromatically with toluidine blue and showed birefringence (Figs. 14 and 15), and responded positively to the Feulgen reaction (Fig. 16). In the slides positioned horizontally, a halo of thin birefringent filaments was present at the nuclear periphery of the spermatozoa and in meiotic chromosomes (Figs. 17 and 18).

DISCUSSION

As shown here, ECFs were produced in mouse sperm cells by the action of gravity after some components of the chromatin, nuclear matrix and nuclear envelope had been disrupted and removed by lysis. ECF formation results from increased chromatin fluidity as a consequence of the breakdown of most DNA/nuclear protein interactions by saline treatment plus the disruption of components of the nuclear envelope by the non-ionic detergent Triton X-100 [5]. The ECFs formed in mouse sperm cells consisted mostly of DNA, as shown by the Feulgen reaction and toluidine blue staining, and by the optical anisotropy following toluidine blue binding [20,21], as well as the practically negative response to acid fast green [3]. These findings differed from those for mouse hepatocytes in which nuclear protein granules were well-identified throughout the ECFs [11]. The anisotropical images seen in ECFs are a function of the highly ordered organization and alignment of the DNA in these fibers [21].

Lysis was more effective in unfixed preparations, although fixation relaxed or removed part of the nuclear protein components from the nuclei [11,15] sufficiently to generate additional free phosphate groups suitable for binding toluidine blue. This binding is responsible for metachromasy (violet color) [8,10,20] in the DNA of mouse spermatozoa.

In fixed, vertically lysed mouse testicular preparations, only part of the spermatozoal population formed ECFs. Even so, the frequency of spermatozoa ECF formation was much higher (60%) than that for hepatocytes under same experimental conditions (maximum, 22%) [11], or even for cells other than spermatozoa present in the testicular preparations. The apparent ease of ECF formation in mouse spermatozoa occurred despite the extremely compact chromatin structure present in these mammalian cells [6]. Although a tightly packed chromatin organization is assumed for DNA-protein complexes containing protamines and protamine-like proteins [1,12], the protocol used here for ECF formation in the presence of a reducing agent is proposed to solubilize protamines for studies of nuclear matrix proteins in rat spermatozoa [16]. This could explain the high frequency of ECF formation in mouse spermatozoa, even in the absence of a reducing agent in the lysis protocol, but could also account for the finding that some of the sperm cells showed no formation of typical ECF, perhaps because the nuclei of these cells contained some protamine-like proteins, as well as a nuclear matrix [6,16]. Even in the sperm cells that did not produce ECFs in fixed and vertically or horizontally lysed preparations, a change in the distribution of the sites responsible for toluidine blue binding, and the presence of a deep birefringent image were detected in the cell nuclei and chromosomes, thus demonstrating an ordered reorganization of the chromatin in response to the different treatments.

The lack of a staining with acid fast green in the center of treated sperm cell nuclei rather than at the nuclear periphery suggests the presence of discrete protamine remnants and nuclear matrix proteins at this location. These observations agree with the consideration that different hierarchies and heterogeneity exist in the packaging of DNA by proteins at different sites in the mammalian sperm cell nucleus [6]. The abundant and prominent reactivity of the nuclear matrix and nucleolar proteins of spermatogonia with acid fast green after lysis and the action of gravity, and the faint staining with acid fast green at the nuclear periphery of spermatozoa, indicate that nuclear matrix proteins change in nature and/or composition during spermatogenesis in the mouse.

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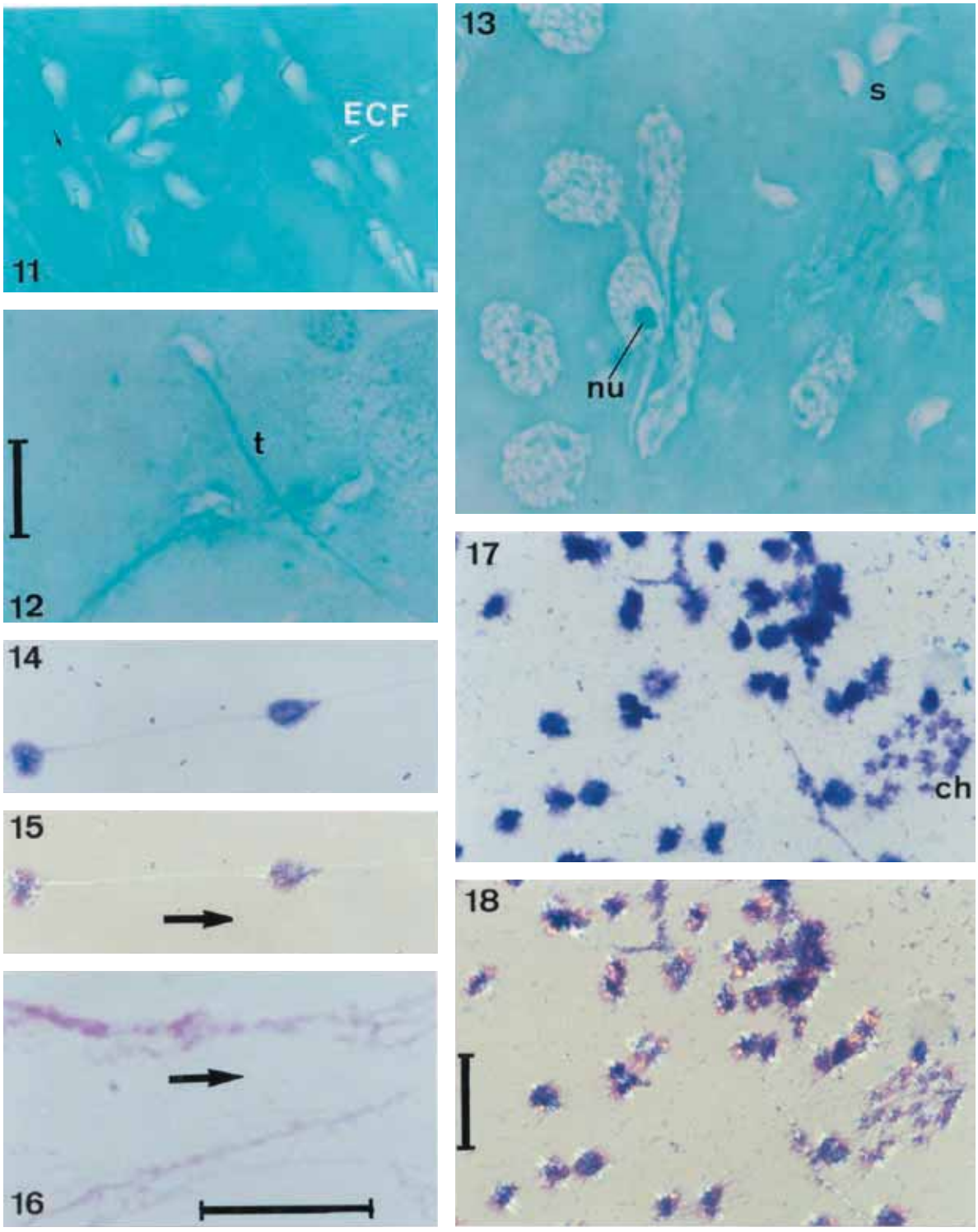

Figures 11-13. Fixed and vertically lysed cell nuclei treated with acid fast green, showing an essentially negative response in sperm ECFs (**11**), and well-stained sperm tails (**t**, **12**), and filaments, granules and a nucleolar-like body (**nu**) inside spermatogonial nuclei (**13**). **s**, spermatozoa.
Figures 14-16. Unfixed and vertically lysed sperm cell nuclei stained with toluidine blue (**14, 15**) and the Feulgen reaction (**16**). **15** is a polarized light view of **14**.
Figures 17 and **18.** Unfixed and horizontally lysed sperm cells treated with toluidine blue. **18** is a polarized light view of **17**, showing a halo of thin birefringent filaments encircling the sperm cell nuclei and meiotic chromosomes (**ch**).
The arrows indicate the direction of gravity. Bars = 20 μm.

REFERENCES

1. Bloch DP (1969) A catalog of sperm histones. *Genetics* **61**, 93-111.
2. 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.
3. Deitch AD (1966) Cytochemistry of nucleic acids. In: *Introduction to Quantitative Cytochemistry* (Wied GL, ed). pp. 451-468. Academic Press: New York.
4. Falco JRP, Mello MLS (1999) Critical electrolyte concentration of spermatozoal chromatin containing histone H1 variants. *Genet. Mol. Biol*. **22**, 197-200.
5. Haaf T, Ward DC (1994) Structural analysis of α-satellite DNA and centromere proteins using extended chromatin and chromosomes. *Hum. Mol. Genet*. **3**, 697-709.
6. Haaf T, Ward DC (1995) Higher order nuclear structure in mammalian sperm revealed by *in situ* hybridization and extended chromatin fibers. *Exp. Cell Res.* **219**, 604-611.
7. Heng HHQ, Squire J, Tsui LC (1992) High-resolution mapping of mammalian genes by *in situ* hybridization to free chromatin. *Proc. Natl. Acad. Sci. USA* **89**, 9509-9513.
8. Mello MLS (1997) Cytochemistry of DNA, RNA and nuclear proteins. *Braz. J. Genet*. **20**, 257-264.
9. Mello MLS, Falco JRP (1996) Critical electrolyte concentration of DNA-protein complexes in spermatozoal and somatic cell nuclei of the honey bee, *Apis mellifera*. *Insect Biochem. Mol. Biol*. **26**, 793-795.
10. Mello ML, Vidal BC (1973) Linear dichroism and anomalous dispersion of birefringence on bee sperm heads. *Acta Histochem*. **45**, 109-114.
11. Moraes AS, Vidal BC, Guaraldo AMA, Mello MLS (2005) Chromatin supraorganization and extensibility in mouse hepatocytes following starvation and refeeding. *Cytometry A* **63**, 94-107.
12. Oliva R, Dixon GH (1991) Vertebrate protamine genes and the histone-to-protamine replacement reaction. *Progr. Nuclei. Acid Res. Mol. Biol*. **40**, 25-94.
13. Perkins TT, Smith DE, Chu S (1997) Single polymer dynamics in an elongational flow. *Science* **276**, 2016-2021.
14. Poirier M, Eroglu S, Chatenay D, Marko JF (2000) Reversible and irreversible unfolding of mitotic newt chromosomes by applied force. *Mol. Biol. Cell* **11**, 269-276.
15. Retief AE, Rüchel R (1977) Histones removed by fixation: their role in the mechanism of chromosomal banding. *Exp. Cell Res*. **106**, 233-237.
16. Santi S, Rubbini S, Cinti C, Squarzoni S, Matteucci A, Caramelli E, Guidotti L, Maraldi NM (1994) Nuclear matrix involvement in sperm head structural organization. *Biol. Cell* **81**, 47-57.
17. Sivak A, Wolman SR (1974) Chromosomal proteins in fixed metaphase cells. *Histochemistry* **42**, 345-349.
18. Strick TR, Allemand JF, Bensimon D, Bensimon A, Croquette V (1996) The elasticity of a single supercoiled DNA molecule. *Science* **271**, 1835-1837.
19. Vidal BC (1972) Doppelbrechungsdispersion und Lineardichroismus von Eu- und Heterochromatin nach Färbung mit Toluidinblau. Nachweis eines Cotton-Effektes. *Beitr. Path*. **145**, 269-285.
20. Vidal BC (1987) Métodos em Biologia Celular. In: *Biologia Celular* (Vidal BC, Mello MLS, eds). pp. 5-39. Livraria Atheneu: Rio de Janeiro.
21. Vidal BC (2000) Extended chromatin fibers: crystallinity, molecular order and reactivity to concanavalin A. *Cell Biol. Int*. **24**, 723-728.
22. Ward WS, Coffey DS (1990) Specific organization of genes in relation to the sperm nuclear matrix. *Biochem. Biophys. Res. Commun*. **173**, 20-25.

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