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EXTENDED CHROMATIN FIBERS IN SPERMATOZOA OF *Apis mellifera* (HYMENOPTERA, APOIDEA)*

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ABSTRACT

The flow of chromatin from the nuclei of mouse liver cells and spermatozoa after treatment with concentrated saline and detergent solutions under the simultaneous action of gravity results in the formation of extended chromatin fibers (ECF). In mouse somatic nuclei, the increase in chromatin condensation is accompanied by a decrease in the frequency of ECF formation. Since tightly packed chromatin with a very lysine-rich histone variant that resembles somatic H1 histones occurs in honey bee spermatozoa, we examined the formation of ECF in sperm cells of *Apis mellifera*, and compared the findings with data for mouse cells. Freshly prepared smears of fixed and unfixed semen from *A. mellifera* were lysed under the action of gravity, stained with toluidine blue at pH 4.0, and examined with polarized and unpolarized light. A protocol using unfixed preparations and a short lysis period that resulted in abundant ECF production in mouse hepatocytes (which contain loosely-packed chromatin) and sperm cells produced ECF in only a few spermatozoa of *A. mellifera*. In contrast, a protocol using fixed preparations and a long lysis period produced fewer ECFs in the former two cell types and no ECF formation in honey bee spermatozoa. The limited chromatin fluidity in *A. mellifera* spermatozoa may reflect their special DNA-protein composition and organization in the cell nuclei, the participation of nuclear matrix elements, a less effective disruption of the nuclear envelope and plasmalemmal components during lysis, and/or cytoplasmic spatial constraints resulting from particularities in the acrosomal complex.

Key words: Chromatin extensibility, histone H1 variant, honey bee, optical anisotropy, spermatozoa

The flow of chromatin from the nuclei of mouse liver cells and spermatozoa, as well as other cell types, after treatment with concentrated saline and detergent solution under the simultaneous action of gravity results in the formation of extended chromatin fibers (ECFs) [5-7,13,14,17,22]. Changes in the chromatin packing states and chromatin viscoelasticity have been found to affect the formation of ECF in mouse liver cells following alterations in cell physiology associated with conditions such as starvation and aging [12,14]. These changes are characterized by an increase in chromatin condensation accompanied by a decrease in the frequency of ECF formation [12,14]. A high frequency of nuclei with ECFs has also been reported in similarly treated mouse sperm cells, and probably results from the extraction of nuclear proteins (in this case, two protamine-like variants) from the DNA-protein complexes of these cells [13].

The DNA-protein complex of honey bee spermatozoa contains a somatic-like, lysine-rich histone H1 variant that differs from the DNA-protein complex of spermatozoa from many other species, the somatic histones of which are partially or totally replaced by protamines (arginine-rich; no lysine), protamine-like variants (arginine-rich, little or no lysine, but oxidized cysteine also present) or intermediate basic sperm nuclear proteins (histidine and/or lysine, in addition to arginine) [1,3,4,9,15,19]. The different chromatin composition and supraorganization of *A. mellifera* spermatozoa

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*Dedicated to Professor Carminda da Cruz-Landim on the occasion of her retirement.

suggests these cells may behave differently from mouse liver cells and spermatozoa when lysed under the action of gravity to induce ECF formation [14]. In this study, ECF formation was investigated in spermatozoa from the semen of drones and from the spermatheca of early-inseminated queens of *A. mellifera*.

MATERIAL AND METHODS

Insects and collection of spermatozoa

Drones and early-inseminated queens of *A. mellifera* L. (Hymenoptera, Apoidea) from colonies maintained at the Institute of Biosciences of Paulista State University at Rio Claro, SP, Brazil, were used. Samples of semen collected from drones immediately after ejaculation following manual manipulation, or from the dissected spermathecae of queens were diluted in cold saline solution and used to prepare smears on glass slides.

Treatments

Freshly prepared smears 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 (to expose them to the action of gravity) and horizontally (a negative control for the action of gravity) and the preparations 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 25ºC, after which the volume of solution was completed with absolute ethanol to a final concentration of 50%, followed by a 10 min incubation. The slides were then removed from the lysis solution and transferred to 70% ethanol for 30 min [6,7,22]. Unfixed preparations subjected to the same lysis protocol but incubated for shorter periods of time (10, 20 and 30 min) were also used. Fixed preparations that had not been subjected to the lysis protocol were used as controls.

Topochemistry and optical anisotropy

Staining was done with 0.025% toluidine blue (Merck, Darmstadt, Germany) in McIlvaine buffer at pH 4.0 for 15 min [21,22]. The preparations were then rapidly (5 s) rinsed in distilled water, air dried, cleared in xylene, and mounted in natural Canada balsam. In addition to being a classic cytochemical method for studying nucleic acids, staining with toluidine blue also allows the investigation of optical anisotropy (birefringence and selective absorption of polarized light, i.e., linear dichroism) of DNA and DNA-protein complexes [10,11,20-22], including ECFs [14,20,22]. These anisotropic characteristics provide information on the suitability and proximity of free DNA phosphates available as binding sites for toluidine blue [10,11,20,21].

The presence of ECFs in the stained nuclei of spermatozoa was assessed with polarized and unpolarized light using a Zeiss Axiophot 2 microscope equipped with a 40/0.75 Pol-Neofluar objective, 1.4 condenser, a compensator and polychromatic light. Birefringence was investigated with a crossed polarizer and analyser by orientating the long axis of the sperm heads at 45º relative to the polarizing azimuths of the analyser and polarizer. Linear dichroism was investigated in the same nuclei using a polarizer and orientating the sperm heads relative to the east-west azimuth of the electrical vector of the polarized light.

RESULTS

The nuclei of *A. mellifera* spermatozoa had a characteristic rod-like shape. Intense metachromasy was seen in control nuclei and in nuclei that were lysed horizontally or vertically and that did not show ECF formation; this metachromasy was more evident after lysis in a horizontal position. The stained nuclei showed intense birefringence with typical yellow/green interference colors (Fig. 1A) and linear dichroism (Fig. 1B,C). The linear dichroism was characterized by a negative sign since the absorption of the stained nuclei positioned perpendicularly to the azimuth of the electrical vector of the polarized light was higher than that of nuclei positioned parallel to the same azimuth (Fig. 1B,C).

There was no ECF formation in fixed preparations subjected to the different lysis protocols. However, toluidine blue-stained chromatin was distributed in a helical pattern within the nuclei and this produced a "banded" image that was especially evident under polarized light (crossed polarizer and analyser with or without a slight compensation for birefringence) (Fig. 2A,B).

ECF formation was seen only in unfixed preparations that were lysed vertically, and involved only some of the spermatozoa (<1%, regardless of the duration of lysis) (Fig. 3A-E). Although the ECFs in these cells consisted of very thin filaments, they were distinguishable from sperm tails by their negative birefringence sign, which is typical of DNA or DNA-protein complexes under polychromatic light (Fig. 3A-C), and by the fact that they stained with toluidine blue at pH 4.0 and showed negative linear dichroism (Fig. 3D,E). The birefringence sign mentioned here is easily demonstrated in Figure 3A-C by considering that when the birefringence in ECFs or in sperm nuclei without ECFs positioned at 45º with respect to the crossed polarizer-analyser

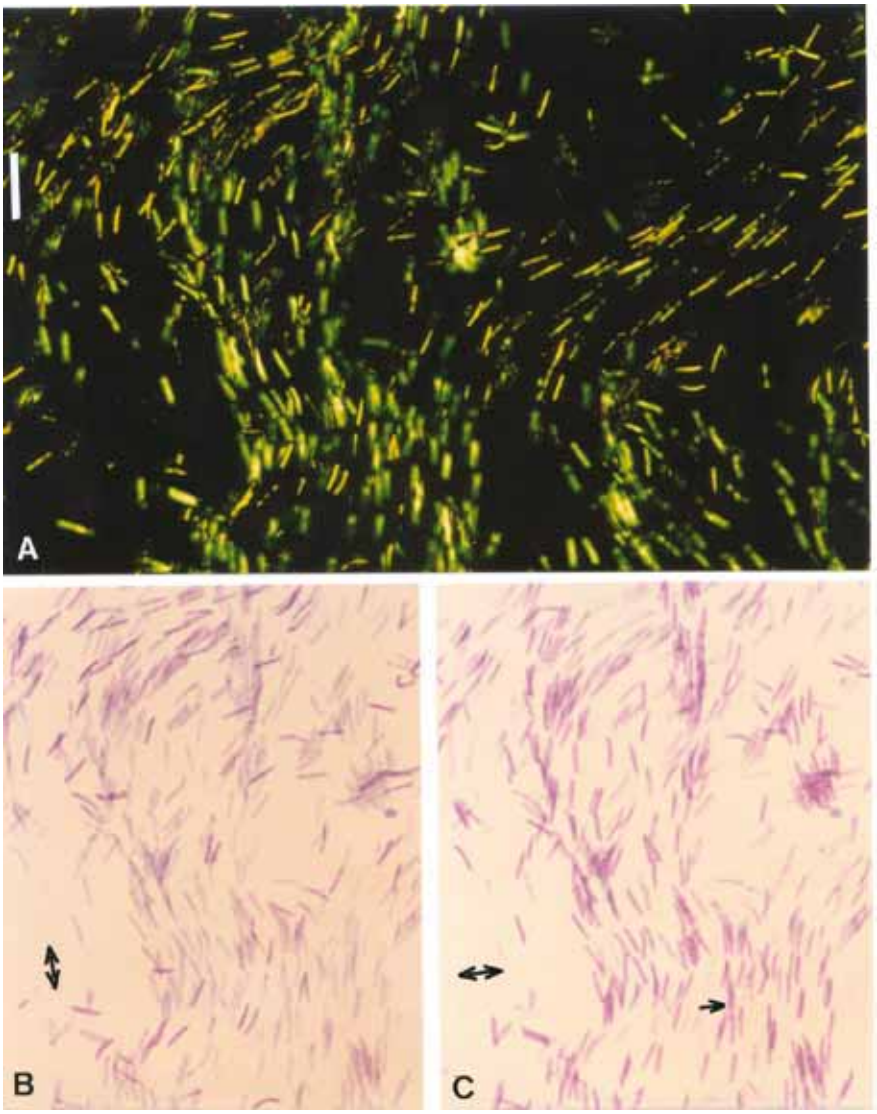

Figure 1. Dispersion of birefringence (**A**) and linear dichroism (**B**,**C**) in nuclei of fixed, toluidine blue-stained spermatozoa of *Apis mellifera*. All images are from the same microscopic field. Absorbances were higher in nuclei positioned perpendicularly (**arrow**) to the azimuth of the electrical vector of polarized light (↔). Bar = 10 μm.

is compensated, the birefringence of nuclei or ECFs positioned at 90º with respect to the former is partially or totally intensified. In some cases, heterogenously distributed, granular, toluidine blue-stained material was seen in ECFs (Fig. 3C).

There were no differences in the nuclear morphology, metachromasy and optical anisotropy among spermatozoa from drones and the spermathecae of queens.

DISCUSSION

The metachromasy and optical anisotropic characteristics of *A. mellifera* spermatozoa under control conditions (linear dichroism and birefringence) agreed with those of a previous report [10]. The present results show that under the experimental conditions used to form ECFs in mouse hepatocytes and sperm cells [13,14,22], ECFs were rarely produced in *A. mellifera* spermatozoa. Indeed, ECF formation was seen only in unfixed semen preparations that were lysed in a vertical position, as previously described [5,7,14,22]. This treatment involves the breakdown of most DNA-histone interactions by the saline components of the solution and disruption of the nuclear envelope components by Triton X-100 [5].

One explanation for the finding that most of the *A. mellifera* spermatozoa did not form ECFs could be that the chromatin involved was very resistant

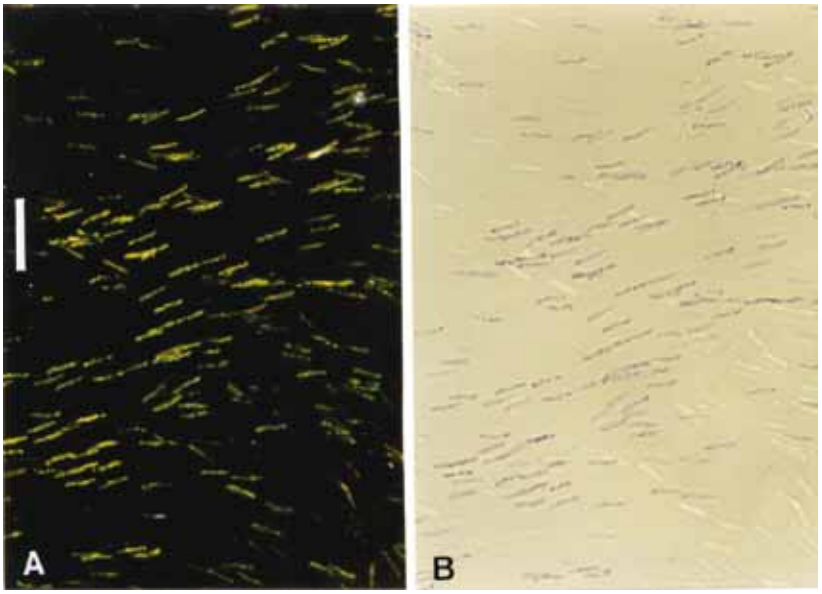

Figure 2. Dispersion of birefringence in nuclei of fixed, toluidine blue-stained spermatozoa of *Apis mellifera* subjected to vertical lysis (**A,B**). Both images were from the same microscopic field. Note the helical distribution of the birefringent stained substrate. In **B,** a slight compensation of the birefringence was introduced to reinforce the "banded" appearance of the birefringent chromatin. Bar = 10 μm.

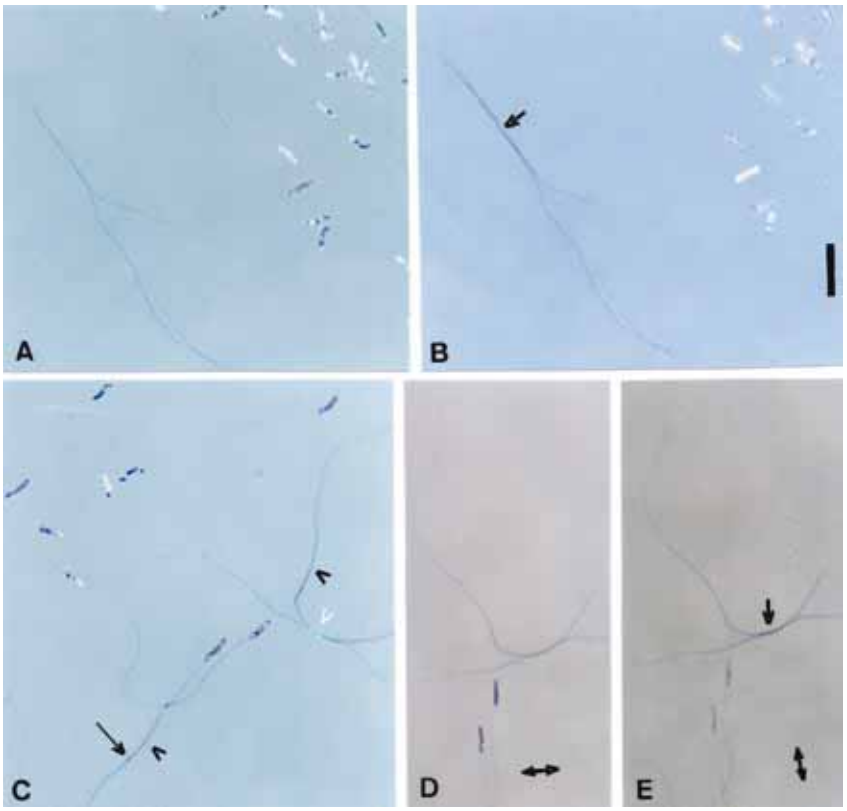

Figure 3. Extended chromatin fibers (ECFs) formed in unfixed spermatozoal nuclei subjected to vertical lysis and toluidine blue staining. The arrow in **B** indicates compensation of the slight birefringence of the ECFs seen in **A**. Total compensation of the birefringence in ECFs (**black arrowhead**) and partial compensation in sperm heads highlighted the birefringence in ECFs (**white arrowhead**) or in part of the sperm heads positioned perpendicularly to the former (**C**). A fine granular distribution of stained material is seen in an ECF (**arrow**) (**C**). Negative linear dichroism was observed in ECFs (**arrow**) and sperm heads positioned differently relative to the azimuth of the electrical vector of polarized light (↔) (**D**,**E**). Bar = 10 μm.

to lysis because of its protein component and its packing state [9,10]. Indeed, the tight packing state of the DNA-protein complex in honey bee spermatozoa could account for the deep electron opacity of the homogeneously distributed chromatin in these cells [2,8]. However, at least part of the histone H1 variant present in *A. mellifera* spermatozoa is removed from the DNA-protein complex during lysis, even when done horizontally, as shown by the increase in nuclear metachromasy and the "banded" distribution of toluidine blue-stained material in the cell nuclei. The "banded" image seen in cell nuclei may represent the helical distribution of chromatin highlighted after lysis. A tandem, end-to-end arrangement of chromosomes, as hypothesized for *A. mellifera* spermatids [Kerr WE, 1969 – personal communication] and observed in *Drosophila* and some Orthoptera [18], could facilitate the production of these "banded" structures after lysis.

Nuclear proteins that could adversely affect ECF fluidity may also be present in the nuclei of *A. mellifera* spermatozoa. Nuclear matrix proteins have been reported in the spermatozoa of other animals [23]. The very fine, granular material that stained with toluidine blue at pH 4.0 in the ECF of *A. mellifera* spermatozoa may correspond to small sites at which the DNA is still not fully stretched, possibly because of some retained nuclear proteins, as also seen in mouse hepatocytes [14]. In the case of mouse hepatocytes, nuclear matrix glycoproteins have been reported to occur in the cell nuclei and granular material of ECFs [14], but there has been no such report for honey bee spermatozoa. Electron microscopy of the nuclei of *Apis* spermatozoa treated with lysis solution in addition to DNA extraction could provide data on the presence of a nuclear matrix. The presence of nuclear glycoproteins could be assessed immunocytochemically by detection with Con-A. Detailed identification of putative nuclear matrix proteins would require extraction of the proteins followed by bidimensional electrophoresis.

An additional explanation for the lack of ECF formation in most *A. mellifera* spermatozoa could be that disruption of the nuclear envelope and plasmalemmal components of these cells was ineffective because of their composition and organization. A cytoplasmic spatial constraint involving the acrosomal complex that, in this species, shows some ultrastructural peculiarities [16], may also be involved.

The presence of DNA in the ECFs of *A. mellifera* spermatozoa was demonstrated by staining with toluidine blue and by the anisotropic characteristics typical for DNA, such as the negative birefringence and linear dichroism in polychromatic light (visible spectrum) following staining with toluidine blue [20,21]. DNA birefringence in the presence of toluidine blue is caused by the orientation of the purine and pyrimidine rings that overlie each other at right-angles to the axial backbone of the macromolecule and by the orientation of the DNA-bound toluidine blue molecules that follow the DNA conformation [10,11,20,21]. The resulting ordered arrangement of toluidine blue molecules attached to the DNA phosphates allows the selective absorption of polarized light [10,11].

Although molecular changes are expected to occur in spermatozoa when they enter the female genital tract, there were no detectable changes in the chromatin fluidity under the experimental conditions used here.

In conclusion, the altered patterns of chromatin fluidity in *A. mellifera* spermatozoa showed here, demonstrate that the ECF formation is a complex process, and it depends on the type of protein the DNA is bound to, and how these macromolecules are complexed, giving to each cell type a specific chromatin organization, according to their physiological functions.

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