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Preliminary study of sperm chromatin characteristics of the brachyuran crab *Maja brachydactyla*. Histones and nucleosome-like structures in decapod crustacean sperm nuclei previously described without SNBPs

K. Kurtz [a,*], J. Ausió [b], M. Chiva [a]

[a] Department of Physiological Sciences II, Faculty of Medicine, University of Barcelona, Barcelona, Spain
[b] Department of Biochemistry and Microbiology, University of Victoria, Victoria, British Columbia, Canada

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ABSTRACT

An interesting characteristic of decapod crustacean sperm nuclei is that they do not contain highly packaged chromatin. In the present study we re-examine the presence of DNA-interacting proteins in sperm nuclei of the brachyuran *Maja brachydactyla*. Although previous reports have indicated that, unlike the majority of sperm cells, DNA of decapod sperm is not organized by basic proteins, in this work we show that: (1) histones are present in sperm of *M. brachydactyla*; (2) histones are associated with sperm DNA; (3) histone H3 appears in lower proportions than the other core histones, while histone H2B appears in higher proportions; and (4) histone H3 in sperm nuclei is acetylated. This work complements a previous study of sperm histones of *Cancer pagurus* and supports the suggestion that decapod crustacean sperm chromatin deserves further attention.

1. Introduction

During spermiogenesis, differentiating spermatids are converted into mature sperm. In most species, this process involves a great reduction of nuclear volume and change in chromatin architecture, producing extreme packaging of the genome. The proper interaction of histones or other sperm nuclear basic proteins (SNBPs) with DNA provokes the progressive condensation of spermiogenic chromatin. The final sperm nucleus possesses chromatin that is very compact, reduced in volume, and with a simplified protein composition. However, the transformation of the spermatid nucleus into the mature sperm nucleus is a complex process that varies in each taxonomic group. This process is particularly interesting in crustaceans, a group of organisms that is the fourth most species diverse (Martin and Davis, 2001). Mature sperm nuclei from several decapod crustacean species are very different from sperm nuclei of other studied taxonomic groups (Yasuzumi, 1960; Moses, 1961; Shigekawa and Clark, 1986; Medina, 1994). Sperm chromatin of some decapods is not at all electron-dense, and appears in a non-compact organization, loosely arranged and suspended in the nucleoplasm (Hinsch, 1969, 1986; Tudge et al., 2001; Medina et al., 2006). Due to the fact that spermiogenesis in many decapod species involves a decondensation of the spermiogenic chromatin (Langreth, 1969; Shigekawa and Clark, 1986), in the past much focus has been placed on describing the nuclear protein transitions and/or basic proteins associated to spermiogenic and mature sperm cells of these organisms, specifically in brachyuran crabs.

The crustacean type of sperm nuclei was first designated by Bloch in a classification of sperm nuclei according to the sperm nuclear basic proteins (SNBPs) interacting with the DNA (Bloch, 1969). Despite several studies dedicated to characterizing this type of sperm (Vaughn and Locy, 1968; Langreth, 1969; Vaughn et al., 1969), the decapod crustacean category of sperm nuclei still remains poorly understood. Around the time of Bloch's sperm classification, sperm nuclei of diverse decapod crustacean species were reported to have no basic proteins associated with DNA, though the alternative stabilizing component of the DNA was never determined. Recently, a study on *Cancer* crabs provided some answers to the longstanding question of how chromatin of crustacean sperm is organized (Kurtz et al., 2008). In this work, micrococcal nuclease digestions of mature sperm chromatin led to the discovery that chromatin is organized by histones into small size nucleosomes; some of these histones appeared to be acetylated or hyperacetylated, in particular, a large portion of histone H4. Additionally, these mature sperm were found to have a much lower basic protein/DNA ratio than other sperm types. In *Cancer*, the low histone/DNA ratio, short linker region of the nucleosomes, and rapid kinetics of

* Corresponding author at: c/Feixa Llarga s/n, Universitat de Barcelona, Facultat de Medicina, Campus Belvitge, Department Ciències Fisiològiques II, Pavelló de Govern, 4ª Planta, Hospitalet de Llobregat 08907. Tel.: +34 93 403 5824; fax: +34 93 402 4268.
E-mail address: klkurt00@smumn.edu (K. Kurtz).

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the chromatin digestion collectively suggest that among regions of sperm chromatin organized by nucleosomes there exist large regions of sperm DNA which are not stabilized by any basic protein. It is supposed that divalent cations are present to stabilize areas without basic proteins (Kurtz et al., 2008).

Many ultrastructural studies have described in detail the morphology of sperm from a variety of decapod species (for extensive reviews, see Jamieson, 1991; Jamieson and Tudge, 2000; Tudge, 2009). The nucleus generally is cup-shaped and extends into radial arms, is positioned in the periphery of the cell, and surrounds a globular, approximately round acrosomal granule, or vesicle (Langreth, 1969; Hinsch, 1988; Chiba et al., 1992; Medina and Rodríguez, 1992; Jamieson, 1994; Jamieson et al., 1998; see also Fig. 2). Additionally, these nuclei exhibit a few particular characteristics that differentiate them from sperm nuclei of many other animals. To begin with, these organelles are often not entirely separated from the cytoplasm by a membrane or envelope (Langreth, 1969; Hinsch, 1986; Tudge et al., 2001); some cytoplasmic structures reminiscent of those observed in spermiogenic cells are found immersed within the chromatin itself, together forming *nucleocytoplasm*. Another characteristic of brachyuran crustacean sperm is its uncondensed, fibrous chromatin (Langreth, 1969; Jamieson et al., 1998; see Fig. 2). This characteristic is quite different than sperm nuclei of other animal species with highly condensed chromatin, a feature which has been interpreted, among other reasons, as a protection of the DNA against mutagenic factors (Subirana, 1975; Braun, 2001).

The peripheral location of the nucleus, as well as its decondensed nature, has made it complicated to purify these nuclei, and therefore, accurately perform biochemical analysis of the chromatin. Previous experiments designed to study the molecular contents of the nucleus of several brachyuran crabs were mostly carried out by specific histochemical staining methods (such as those used in Alftert and Geschwind, 1953), though an attempt was made to solubilize the chromatin using harsh mechanical methods to analyze the solublized proteins (Vaughn and Hinsch, 1972). Chevaillier (1966, 1967, 1968) described that in *Eupagurus bernhardus* and *Carcinus maenas* the histones migrate from the nucleus to the acrosomal granule in the last steps of spermiogenesis, while Vaughn and Hinsch (1972) describe only acidic proteins associated to sperm DNA of the majid *Libinia emarginata*. Later, species of the genus *Cancer* were studied by Langreth (1969), who concluded that all histones apparently leave the nucleus in the most advanced steps of spermiogenesis; but a discrepancy to these findings was described in the recent re-examination of sperm chromatin of *Cancer* crabs (Kurtz et al., 2008).

Due to great interest in understanding the biochemical nature and organization of the chromatin in decapod crustacean sperm, we have re-examined the basic protein composition of sperm chromatin of the brachyuran crab *M. brachydactyla*. This species is an interesting model, considering sperm of several majid species have been used in earlier ultrastructural studies (Hinsch, 1969, 1971; Chiba et al., 1992; Tudge and Justine, 1994; Jamieson et al., 1998), and have also been portrayed as void of basic DNA-interacting proteins (Vaughn and Hinsch, 1972).

2. Material and methods

2.1. Animals

Male individuals of *M. brachydactyla* and *Cancer pagurus* (both decapod brachyuran crabs) were purchased live from markets in Barcelona, Spain, and identified according to their morphological characteristics. The vasa deferentia ducts containing mature sperm within spermatophores were removed. Male individuals of the bony fish *Sparus aurata* were purchased live from fishermen in Barcelona, Spain, and male echinoderms *Holothuria tubulosa* were obtained off the Mediterranean coast of Catalonia, Spain. The sperm were isolated from each species. Sperm were washed with ice-cold buffer A (0.25 M sucrose, 10 mM Tris pH 7.4, 3 mM $MgCl_2$, 5 mM $CaCl_2$, 0.1 mM spermine, 0.25 mM spermidine), containing either 25 mM benzamidine chloride or proteolytic inhibitor cocktail tablets (Roche Diagnostics) to inhibit proteolysis.

2.2. Transmission electron microscopy (TEM)

Small sections of testes and vasa deferentia ducts were dissected from live male individuals of *M. brachydactyla* and were fixed in a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 for 24 h at 4 °C. Samples were rinsed in cacodylate buffer (3 times for 10 min and 3 times for 30 min) and post-fixed for one and a half hours at 4 °C in 1% osmium tetraoxide in cacodylate buffer. The samples were then rinsed in cacodylate buffer twice for 5 min and once for 30 min. TEM samples were then dehydrated using an increasing acetone series (30% for 15 min, 50% for 15 min, 70% for 15 min, 70% overnight at 4 °C, 90% for 60 min, 95% for 75 min, and twice 100% for 30 min) and embedded in Spurr's resin. Ultra-thin sections were made using a Leica UCT ultramicrotome, and counterstained with uranyl acetate and lead citrate. Observations were made on a Jeol EM-1010 transmission electron microscope at 80 kV.

2.3. Obtaining purified sperm nuclei or free sperm cell populations

The purification of sperm cell nuclei from *S. aurata* and *H. tubulosa* was performed as previously described in Giménez-Bonafé et al. (2004). To obtain free sperm cells from *M. brachydactyla*, the deferential ducts were gently homogenized manually in buffer A, and filtered through 4 layers of gauze. The filtrate (containing spermatophores and free sperm cells released from spermatophore capsules) was stirred during 15 min at 4 °C to free sperm cells from spermatophores. Free sperm cells were separated from spermatophore capsules and full spermatophores by sedimentation at unit gravity through a discontinual sucrose gradient of 0.25 M sucrose, 1 M sucrose, and 2.2 M sucrose. The denser material, being full spermatophores and spermatophore capsules, sunk to the interphase of 1 M sucrose and 2.2 M sucrose. After allowing full separation of free sperm from spermatophores in the sperm cell suspension, the top layers containing free sperm suspended in 0.25 M/1 M sucrose were collected and washed in 6 volumes of buffer A. All procedures were performed at 4 °C. From this point, the sperm cells released from spermatophores were used for micrococcal nuclease digestions or extraction of basic proteins.

2.4. Extraction of nuclear proteins

Several types of protein extractions were attempted, using sperm obtained from various individuals on independent occasions. Total protein extraction was performed with 2% SDS, 60 mM Tris, 0.7 M β-mercaptoethanol, and 5 mM EDTA; separate extractions in buffer containing 6 M urea, 20 mM Tris pH 8.0, and 8% β-mercaptoethanol, or with 0.4 N H_2SO_4, were applied as well (results not shown). In all cases the relative amount of soluble histone proteins was the same; however, additional proteins were solubilized in some buffers which overpowered and masked the histone bands by comparison. Ultimately, the following form of protein extraction was determined to be the most appropriate for protein analysis: Free sperm cells were pelleted and total basic proteins were extracted with 5 volumes of 0.4 N HCl; histone H1 and related proteins were extracted with 5% perchloric acid (PCA). Sol-