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Nuclear size as estrogen-responsive chromatin quality parameter of mouse spermatozoa

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ABSTRACT

Recently, we have investigated the endocannabinoid involvement in chromatin remodeling events occurring in male spermatids. Indeed, we have demonstrated that genetic inactivation of the cannabinoid receptor type 1 (*Cnr1*) negatively influences chromatin remodeling mechanisms, by reducing histone displacement and indices of sperm chromatin quality (chromatin condensation and DNA integrity). Conversely, *Cnr1* knock-out ($Cnr1^{-/-}$) male mice, treated with estrogens, replaced histones and rescued chromatin condensation as well as DNA integrity. In the present study, by exploiting $Cnr1^{+/+}$, $Cnr^{+/-}$ and $Cnr1^{-/-}$ epididymal sperm samples, we show that histone retention directly correlates with low values of sperm chromatin quality indices determining sperm nuclear size elongation. Moreover, we demonstrate that estrogens, by promoting histone displacement and chromatin condensation rescue, are able to efficiently reduce the greater nuclear length observed in $Cnr1^{-/-}$ sperm. As a consequence of our results, we suggest that nucleus length may be used as a morphological parameter useful to screen out spermatozoa with low chromatin quality.

1. Introduction

Spermatozoa (SPZ) are highly differentiated cells with a particular chromatin organization that results from remodeling events occurring in elongating and condensing spermatids (SPT) (Kierszenbaum and Tres, 2004). Indeed, during the post-meiotic stage of spermatogesis, when round SPT are extensively remodeled to form mature SPZ, a gradual and radical change in the chromatin cytoarchitecture is observed (Marcon and Boissonneault, 2004). This radical change requires (i) expression and storage of specific proteins involved in condensation, (ii) displacement and degradation of the nucleosomal structure, (iii) sequential histone replacement by transition proteins and afterwards by protamines, (iv) transcriptional silencing and DNA repair, (v) repackaging of protaminated chromatin into toroidal structures (Oliva and Castillo, 2011). However, many species retain a small fraction (1% in mouse, 15% in human) of their chromatin in the more relaxed nucleosomal configuration so that SPZ contain at least two differentially packaged chromatin domains: (1) the protamine-based chromatin that organizes the bulk of DNA in a highly compact toroidal configuration, suitable to arrest transcription and mask genome from exogenous and endogenous damage until fertilization (Carrell et al., 2007); (2) the nucleosome-based chromatin that organizes epigenetically marked developmental loci in a potentially dynamic transcriptional configuration useful after fertilization (Brykczynska et al., 2010; Miller et al., 2010). Abnormal sperm histone or protamine content can disrupt chromatin organization. Indeed, histone retention decreases nucleoprotamine-based chromatin and exposes a more relaxed chromatin to damage (Carrell et al., 2007; Chioccarelli et al., 2010). In both humans and animals, abnormal DNA damage is associated with compromised fertility and increased miscarriage rates (Carrell et al., 2007; Lewis and Agbaje, 2008; Zhao et al., 2004). Therefore, chromatin quality is an objective marker of sperm function that provides a significant prognostic factor for male infertility (Agarwal and Said, 2003). At morphological level, when histone-to-protamine transition occurs, an extraordinary event is observed in the nucleus of differentiating germ cells: flocculent densities of chromatin coalesce into a coarsely granulo-fibrillar chromatin, which gradually extends in a centripetal and rostral-to-caudal direction and becomes dense and homogeneous at the end of spermiogenesis (Dadoune, 2003). This chromatin remodeling modifies the shape of the whole nuclear compartment and strongly reduces its size promoting development of the peculiar elongated, small and hydrodynamic sperm head that supports swimming ability. By staking these toroids, the sperm nucleus achieves a higher efficiency in packaging the

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paternal genome and therefore in reducing its size to an absolute minimum (Miller et al., 2010). The mechanism by which protamines induce the conformational change in chromatin packaging is not well understood but it is probably related to protamine properties and enzymes involved in chromatin remodeling (Carrell et al., 2007). Histones and protamines are highly basic proteins, characterized by lysine and arginine/cysteine residues, respectively (Oliva, 2006). Protamines have a greater affinity for DNA, because of a higher hydrogen binding potential of arginine over lysine (Helene et al., 1982). These proteins may bind to the major and minor groove of DNA or to the DNA surface by interacting electrostatically with phosphate residues. It has been demonstrated that protamines allow chromatin condensation through arginine residues into toroidal structures at testicular level, and further through cysteine residues along the epididymal transit, when inter- and intra-protamines disulfide bonds are formed (Balhorn et al., 1992).

In humans, the microscopic examination of sperm morphology shows that this complex morphogenetic process is not very efficient, generating a heterogeneous subpopulation of SPZ in the ejaculate with various abnormal and imperfect features. The causes of these imperfections and the possible consequences for fertility are matter of more and more investigations. Indeed, innovative methods for the selection of SPZ in assisted reproduction techniques (ART) have been investigated, providing fresh insight into the correlation between morphological parameters of SPZ and clinical results (Bartoov et al., 2002).

Endocannabinoids are lipidic mediators identified in several tissues (brain, testis, epididymis) and biological fluids (follicular fluid, maternal milk, blood) (Cobellis et al., 2006, 2010; Devane et al., 1992; Habayeb et al., 2004; Schuel et al., 2002; Sugiura et al., 1996). They regulate reproduction, in both males (Battista et al., 2012; Cacciola et al., 2008a, 2010; Chianese et al., 2011, 2012; Fasano et al., 2009; Francavilla et al., 2009; Lewis and Maccarrone, 2009; Lewis et al., 2012a,b; Maccarrone et al., 2003, 2005a; Meccariello et al., 2006, 2008; Pierantoni et al., 2009a; Sun et al., 2009) and females (Acone et al., 2009; Cacciola et al., 2010; Lazzarin et al., 2004; Maccarrone et al., 2005b; Sun and Dey, 2012; Trabucco et al., 2009; Wang et al., 2007), and specific cannabinoid receptors (Cnr1 and Cnr2) have been localized in male and female reproductive tracts (Grimaldi et al., 2009; Karasu et al., 2011; Pertwee et al., 1996; Pierantoni et al., 2009b). In the testis, Cnr1 is present in somatic and germ cells including SPT and SPZ (Barbonetti et al., 2010; Barboni et al., 2011; Bernabò et al., 2012; Cacciola et al., 2008a; Catanzaro et al., 2011; Cobellis et al., 2006; Gye et al., 2005; Maccarrone et al., 2003; Rossato et al., 2005). In rat, Cnr1 appears in round SPT around the nucleus where acrosome is forming. The signal is retained in the head of elongating SPT, always close to the acrosome region, supporting the involvement of Cnr1 in the acrosome and head shape configuration (Cacciola et al., 2008a, 2008b; Maccarrone et al., 2005a). Recently, we have characterized the reproductive phenotype of *Cnr1* knock-out mice (*Cnr1*$^{-/-}$) and reported that males show normal progression of spermatogenesis (Cacciola et al., 2008a; Ricci et al., 2007; Cobellis et al., 2010) and retained fertility, although possessing high percentage of qualitatively poor SPZ with immature chromatin (Cacciola et al., 2013; Chioccarelli et al., 2010). Fertility may be ascribed to the presence of a heterogeneous population of SPZ with mature and immature chromatins. Indeed, in *caput* epididymis, the number of SPZ with abnormal histone retention as well as the number of SPZ with uncondensed chromatin or with DNA damage was higher compared to *Cnr1*$^{+/-}$ and *Cnr1*$^{+/+}$ animals (Chioccarelli et al., 2010).

In the current study, exploiting *Cnr1*$^{+/+}$, *Cnr1*$^{+/-}$ and *Cnr1*$^{-/-}$ sperm samples, we carried out a correlation analysis between histone retention and chromatin condensation or DNA damage with the aim to verify if histone retention might be used as an index of poor sperm chromatin quality, and if it deranged the nuclear size of SPZ. As head shape and size are known to affect sperm motility and function (Malo et al., 2006; Miller et al., 2010; Ostermeier et al., 2001), it is likely that nuclear dimension is an important requisite to achieve optimal head shape and that efficient compaction of the paternal genome facilitates this optimization.

2. Materials and methods

2.1. Experimental animals

CD1-WT (*Cnr1*$^{+/+}$) male mice or males carrying a *Cnr1*-null mutation (Ledent et al., 1999), either in heterozygous (*Cnr1*$^{+/-}$) or homozygous (*Cnr1*$^{-/-}$) condition, were used in this study. Heterozygous mice were bred on a CD1 background (Charles River Laboratories, Lecco, Italy) before generating male mice. All animals were maintained on a standard pellet diet with free access to water. Adult males (4–8 months) were killed by cervical dislocation and epididymides were processed for SPZ sampling. Each experimental analysis included at least 4 different animals for each genotype or experimental group and each animal was analyzed in duplicate.

Experiments were approved by the Italian Ministry of Education and the Italian Ministry of Health. Procedures involving animal care were carried out in accordance with National Research Council's publication *Guide for Care and Use of Laboratory Animals* (National Institutes of Health Guide).

2.2. In vivo and in vitro experiments with 17-β Estadiol (E_2)

In vivo experiment. *Cnr1*$^{-/-}$ males mice ($n = 16$ divided in four groups) of 24 days post partum (dpp) were injected as follows: vehicle (1% ethanol, group 1); E_2 (1.5 μg/100 g dose for each injection, group 2); E_2 (1.5 μg/100 g dose for each injection) plus the estrogen receptor antagonist ICI182780 (ICI; 15 μg/100 g dose for each injection, group 3) and ICI alone (15 μg/100 g dose for each injection, group 4). All the substances were dissolved in ethanol, diluted in physiological solutions (100 μl containing 1% ethanol) and injected *intraperitoneum* for 7 weeks on alternate days. At the end of this period, animals were killed by cervical dislocation. Epididymides were collected and immediately processed for SPZ sampling and aniline blue- or propidium iodide-(PI) stained SPZ slide preparations. The pharmacological treatment was performed on 24 dpp mice according to the presence of round SPT and was halted 7 weeks later, because the first wave of spermatogenesis and SPZ transfer to epididymis lasted about 60 days. This time window, as well as the doses and method of $E_2 \pm$ ICI administration, were chosen to evaluate E_2 effects on chromatin remodeling of SPT as previously reported (Cacciola et al., 2013).

In vitro experiment. Epididymal SPZ samples ($n = 8$ divided in five groups) collected from adult *Cnr1*$^{-/-}$ mice ($n = 4$) were incubated in PBS for 1 h with vehicle (0.03% ethanol according to relative compound concentrations, group 1) or with different E_2 concentrations (10 or 100 nM, groups 2 and 3) ± ICI (100 or 1000 nM according to E_2 concentrations, groups 4 and 5). Further groups were incubated with ICI alone (100 or 1000 nM, groups 6 and 7). Ethanol (0.03%) was added in each experimental group. E_2 was used at the doses which have been demonstrated to have nongenomic effects on sperm function (Ded et al., 2010), since SPZ are transcriptionally inactive cells. ICI was always added 30 min before E_2 and at a concentration 10-fold higher than E_2 to efficiently counteract the hormone effect, as previously reported (Cobellis et al., 2002, 2008).