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

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A B S T R A C T

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.

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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$_2$ ± 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).

At the end of treatment, SPZ were dried on polylysine slides and processed for aniline blue or PI staining. The experiment was replicated three time.

2.3. Epididymal SPZ sampling and Sonication-Resistant-sperm Nuclei (SRN) preparation

Epididymides were dissected, immersed in PBS (pH 7.6) and cut to let the SPZ flow out from the ducts as previously reported (Cobellis et al., 2010). The SPZ samples were then filtered throughout cheesecloth and processed for different staining (aniline blue, acridine orange, PI) and for SRN preparation (Ferrara et al., 2013; Zhao et al., 2001). Briefly, SRN fraction was prepared as previously reported: SPZ were resuspended in TNE [0.1 M Tris–HCl pH7.6, 0.15 M NaCl, 1 mM EDTA pH 8] buffer containing protease inhibitors (2 µg/ml of leupeptin, aprotinin, pepstatin A, and chymostatin; and 5 µg/ml of tosyl phenylalanyl chloromethyl ketone, 0.5 mM phenylmethylsulfonyl fluoride) and sonicated on ice for 1 burst × 15 s with a mild 40% output power. SRN were finally separated from debris in 50% sucrose cushion (1 ml) by centrifugation at $8000 \times g$ for 60 min at 4 °C, washed two time in TNE buffer and used for aniline blue-stained SRN slide preparation. Such a sonication method removes plasma membrane, acrosome and tail without altering chromatine structure. Indeed, it has been demonstrated that this approach does not modify acridine orange staining properties when intact sperm cells or sonicated sperm nuclei were measured (Evenson et al., 1991; Zhao et al., 2001).

2.4. Aniline blue staining

Aniline blue dye shows selective high affinity for lysine and is able to discriminate immature SPZ, characterized by immature chromatin with lysine-rich histones, from mature SPZ characterized by mature chromatin with arginine-/cysteine-rich protamines (Agarwal and Said, 2003). Therefore aniline blue dye provides direct and indirect information about histone and protamine content, respectively. We exploited this property to evaluate histone content and nuclear size of immature and mature SPZ.

Epididymal SPZ (n = 4–5 for each genotype or experimental group) and SRN ((n = 4; samples were collected independently by genotype) samples were dried on polylysine slides, fixed in 4% paraformaldehyde for 10 min at 4 °C, stained with aniline blue staining solution [5% aqueous aniline blue (Sigma–Aldrich) mixed with 4% acetic acid (pH 3.5)] for 7 min and washed. After washing, slides were dehydrated and mounted: epididymal SPZ slides were used for histone retention analysis while SRN slides were used for nuclear size evaluation.

2.5. Acridine orange staining

Epididymal SPZ samples (n = 5 for each genotype) were suspended in 1 ml of ice-cold PBS (pH 7.4) buffer ($1 \times 10^6/100$ µl) and centrifuged at $600 \times g$ for 5 min. The pellet was resuspended in ice-cold TNE buffer [0.01 M Tris–HCl, 0.15 M NaCl, and 1 mM EDTA (pH 7.4)] and centrifuged again at 600xg for 5 min. The pellet was then resuspended in ice-cold TNE buffer with 10% glycerol (200 µl) and immediately fixed in ethanol (70% vol/vol) at 4 °C at least for 24 h. For acridine orange staining, samples were treated with 400 µl of a solution of 0.1% Triton X-100, 0.15 M NaCl and 0.08 N HCl (pH 1.2). After 30 s, 1.2 ml of staining buffer [6 µg/ml acridine orange, 37 mM citric acid, 126 mM Na_2HPO_4, 1 mM disodium EDTA, 0.15 M NaCl (pH 6.0)] was added to the test tube and all samples were simultaneously analyzed by flow cytometry. After excitation by a 488-nm wavelength light source, acridine orange bound to a double-stranded DNA fluoresced green (515–530 nm), and acridine orange bound to a single-stranded DNA fluoresced red (630 nm or greater). As previously described (Chioccarelli et al., 2010), we took advantage of this metachromatic shift, from green to red, to analyze 1) the percentage of SPZ with high DNA stainability (HDS, index of uncondensed chromatin, since uncondensed chromatin was highly accessible to the dye) calculated by ratio intensely green ($FL1 > 10^3$)/total green ($FL1 > 10^1$) fluorescing SPZ; 2) the percentage of SPZ with DNA damage (DD, index of DNA damage) calculated by ratio red fluorescing ($FL3 > 10^1$) /total fluorescing (red plus green; $FL3 > 10^1 + FL1 > 10^1$) SPZ. A minimum of 10,000 cells for each sample were analyzed by fluorescent activated cell sorting (FACSCalibur; BD BioScience, Milan, Italy).

2.6. PI staining

Epididymal SPZ samples (n = 4 for each genotype or experimental group) were dried on polylysine slides, fixed in 4% paraformaldehyde for 10 min at 4 °C and stained for 2 min with PI (0.5 µg/ml; Sigma–Aldrich), a red-fluorescent intercalating agent. After washing, PI-stained SPZ slides were immediately used for sperm nuclear size evaluation. Fluorescence provided an optimal contrast useful to define nuclear outline.

2.7. Histone retention analysis

Sperm histone content and, indirectly, protamine content can be easily evaluated through acidic aniline blue staining (Agarwal and Said, 2003). Indeed, aniline blue dye selectively stains persisting lysine-rich histones, thus allowing the visualization of immature SPZ with defective chromatin as poorly protamined and condensed (Terquem and Dadoune, 1983). Conversely, mature sperm containing arginine-/cysteine-rich protamines lose their ability to be stained with aniline blue dye and appear unstained (i.e. white/gray nuclei) (Dadoune and Alfonsi, 1986).

Aniline blue-stained SPZ slides (n = 4 for each genotype or experimental group) were used to evaluate histone retention. A total of 200 SPZ for each slide were evaluated under a light microscope (Leica Microsystems Inc., Milan Italy) at ×1000 total magnification (×100 oil objective combined with ×10 ocular lens). As above reported, three classes of SPZ with stained nuclei were observed: white/gray (unstained; hereafter named white SPZ), light blue (slightly stained; hereafter named light blue SPZ,) and dark blue (strongly stained; hereafter named dark blue SPZ). Light and dark blue staining were considered as an index of low and high amount of retained histones, respectively (Agarwal and Said, 2003; Chioccarelli et al., 2010). White, light blue and dark blue SPZ were counted, expressed in percentage and reported as mean values ± SD. As routinely required for this experimental procedures, all the results were validated two time by the same operator.

2.8. Sperm nuclear size evaluation by a computer-assisted measurement

PI-stained SPZ (n = 5 slides for each genotype or experimental group) or aniline blue-stained SRN (n = 5 slides, collected independently by genotype) were observed under a light microscope (Leica Microsystems Inc., Milan Italy) at ×1000 final magnification (×100 objective plus ×10 ocular). Images were captured using a high resolution digital camera (DC300F; Leica Microsystems Inc., Milan, Italy) and width and length of each nucleus were analyzed through the IM100 software using a measurement module tool (Leica Microsystems Inc., Milan, Italy). For each slide, the most of SPZ/SRN were considered not eligible for the analysis; indeed, we specifically selected and measured the SPZ/SRN (n = 50 for each slide) whit dorsal angle and broad sides of their area in the plane of focus (see inset of Fig. 2). As routinely required for this experimental procedures, all the results were validated two times by the same operator.
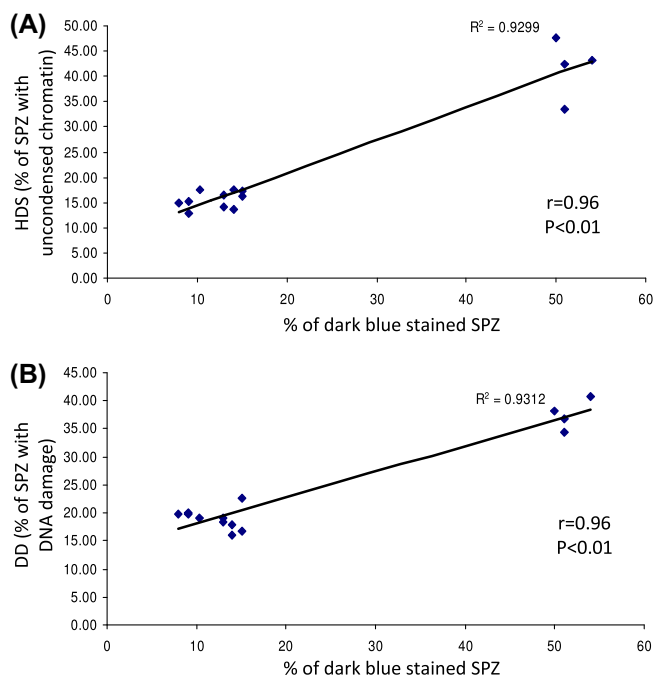
Fig. 1. Correlation analysis between the percentage of dark blue SPZ and the percentage of SPZ with uncondensed chromatin (A) or with DNA damage (B). R^2: coefficient of determination; r: pearson product-moment correlation coefficient; P: probability. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Nuclear dimensions (width and length) of PI-stained SPZ were analyzed accordingly with the genotype or experimental group and reported as mean values ± SD. Width and length of aniline blue-stained SRN were analyzed accordingly with histone retention and reported as mean values ± SD of white, light blue and dark blue nuclei.

2.9. Correlation curves and statistical analysis

The collected data were compared using the Excel built-in distribution functions available in Microsoft Office. The value of r

was considered to establish the test significance. The range $-1 \leqslant r \leqslant 1$ established negative or positive correlation between histone retention (i.e. percentage of dark blue SPZ) and HDS (i.e. percentage of SPZ with uncondensed chromatin) or DD (i.e. percentage of SPZ with DNA damage).

The scatter diagram (length–width) was performed to calculate the threshold value that could exclude at least 95% of the sperm nuclei with bad chromatin features ("bad" SPZ); this threshold was equivalent to a straight line with general equation: nuclear length + width = K, where K was such a value that less of 5% of all the sums (nuclear length + width) of the "bad" SPZ were smaller than K. By this way, finding a SPZ with its nuclear size (length + width) smaller than K (i.e. below the 5th percentile), there was a $P < 0.05$ that it was a "bad" SPZ.

ANOVA followed by Duncan's test for multigroup comparison were conducted to identify groups having different mean. Data are expressed as mean ± SD from at least three or four independent experimental procedures.

3. Results

3.1. Characterization of sperm chromatin and correlation analysis

We exploited the specific properties of aniline blue and acridine orange dyes to analyze chromatin features (histone retention, chromatin condensation and DNA damage) of epididymal SPZ collected from $Cnr1^{+/+}$, $Cnr1^{+/-}$ and $Cnr1^{-/-}$ animals. Correlation analysis between histone retention and uncondensed chromatin or DNA damage was our final goal.

Results of histone retention are reported in Table 1. Data confirm previous observations reported on SPZ collected from caput epididymis (Chioccarelli et al., 2010) and show that the percentage of white SPZ was high in $Cnr1^{+/+}$ (72.12 ± 1.61), significantly lower in $Cnr1^{+/-}$ (59.97 ± 1.77) and further decreased in $Cnr1^{-/-}$ (20.52 ± 1.61) SPZ samples ($P < 0.01$). Conversely, the percentage of dark blue SPZ was high ($P < 0.01$) in $Cnr1^{-/-}$ (49.61 ± 1.52), significantly lower in $Cnr1^{+/-}$ (13.31 ± 1.22) and further decreased in $Cnr1^{+/+}$ (8.55 ± 0.80) SPZ samples. Furthermore, the percentage of light blue SPZ was similar in $Cnr1^{-/-}$ (29.87 ± 3.05) and $Cnr1^{+/-}$ (29.71 ± 1.76), but lower values were detected in the $Cnr1^{+/+}$ (19.32 ± 1.22; $P < 0.01$) SPZ samples. Results of chromatin
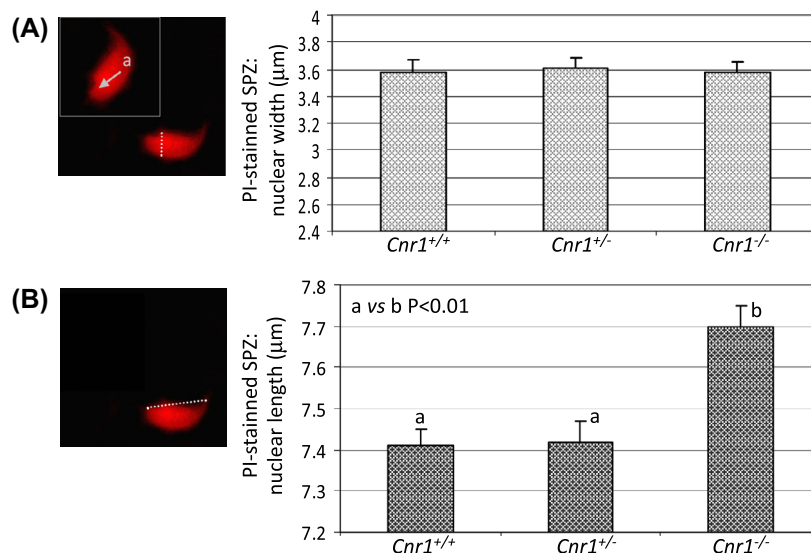

Fig. 2. Nuclear width (A) and length (B) of PI-stained SPZ from $Cnr1^{+/+}$, $Cnr1^{+/-}$ and $Cnr1^{-/-}$ animals. Results are reported as mean values ± SD. The lateral images indicate how SPZ nuclei dimensions have been estimated. a = dorsal angle.

Table 1
Analysis of aniline blue stained-SPZ from $Cnr1^{+/+}$, $Cnr1^{+/-}$ and $Cnr1^{-/-}$ mice: three classes of stained nuclei were observed and reported as mean percentage of white, light blue and dark blue SPZ ± SD. a, b, c, d, e, f, g, h $P < 0.01$.

	White (%)	Light blue (%)	Dark blue (%)
$Cnr1^{+/+}$	72.12 ± 1.61^a	19.32 ± 1.22^d	8.55 ± 0.80^f
$Cnr1^{+/-}$	56.97 ± 1.77^b	29.71 ± 1.76^e	13.31 ± 1.22^g
$Cnr1^{-/-}$	20.52 ± 1.61^c	29.87 ± 3.05^e	49.61 ± 1.52^h

Table 2
Analysis of acridine orange-stained SPZ from $Cnr1^{+/+}$, $Cnr1^{+/-}$ and $Cnr1^{-/-}$ mice. Mean values of HDS (percentages of SPZ with High DNA Stainability/uncondensed chromatin) and DD (percentages of SPZ with DNA Damage) ± SD. a, b, c, d $P < 0.01$.

	HDS (%)	DD (%)
$Cnr1^{+/+}$	15.14 ± 1.89^a	19.65 ± 0.41^c
$Cnr1^{+/-}$	15.83 ± 1.64^a	18.48 ± 2.33^c
$Cnr1^{-/-}$	41.62 ± 5.91^b	37.53 ± 2.64^d

Table 3
Analysis of aniline blue-stained SPZ from $Cnr1^{-/-}$ animals treated *in vivo* with vehicle (Control) or with E2 ± ICI in comparison with untreated $Cnr1^{+/+}$ mice. Three classes of stained sperm nuclei were observed and reported as percentage of white, light blue and dark blue. a, b, c, d $P < 0.01$.

		White (%)	Light blue (%)	Dark blue (%)
$Cnr1^{+/+}$		70.30 ± 1.50^a	20.97 ± 1.02^b	9.10 ± 0.68^d
$Cnr1^{-/-}$	Control	19.85 ± 1.27^b	29.54 ± 1.32^c	50.62 ± 0.46^c
	E_2	71.48 ± 1.70^a	21.40 ± 1.61^b	11.13 ± 1.62^d
	$E_2 + ICI$	19.43 ± 2.30^b	30.85 ± 1.30^c	49.73 ± 2.31^c
	ICI	19.72 ± 1.7^b	30.70 ± 1.29^c	49.70 ± 0.99^c

found when the analysis was carried out using light blue SPZ (data not shown).

3.2. Nuclear size evaluation of PI-stained SPZ and aniline blue-stained SRN

Nuclear width and length of SPZ from Cnr1$^{+/+}$, Cnr1$^{+/-}$ and Cnr1$^{-/-}$ animals were evaluated, independently by histone content, using PI-stained SPZ slides. Fig. 2 shows that nuclear width was not statistically different between $Cnr1^{+/+}$ (3.58 ± 0.09 m), $Cnr1^{+/-}$ (3.60 ± 0.08 μm) and $Cnr1^{-/-}$ (3.58 ± 0.08 μm) animals (panel A). Conversely, nuclear length was significantly ($P < 0.01$) higher in $Cnr1^{-/-}$ (7.72 ± 0.05 μm) than in $Cnr1^{+/-}$ (7.42 ± 0.05 μm) and $Cnr1^{+/+}$ (7.41 ± 0.04 μm) animals (panel B). We hypothesized that this increased length was related to the high number of dark blue-SPZ (with abnormal histone amount) produced by Cnr1$^{-/-}$ mice Therefore, we measured nuclear size according to histone content and decided to carried out this analysis on aniline blue-stained SRN. Indeed, in order to avoid any possible mistakes due to acrosome presence, we preferred to detach nuclei from heads by sonication since this method destroys plasma membrane, acrosome and tail without affecting chromatin condensation or DNA integrity (Evenson et al., 1991; Zhao et al., 2001). Fig. 3 shows that the mean width (panel A) was not statistically different between white (3.37 ± 0.08 μm), light blue (3.37 ± 0.03 μm) and dark blue SRN (3.35 ± 0.07 μm). Conversely, the mean length (panel B) was high in dark blue SRN (7.81 ± 0.07 μm), significantly ($P < 0.01$) lower in the light blue (7.50 ± 0.04 μm) and further decreased ($P < 0.01$) in the white ones (7.34 ± 0.06 μm).

Similar results were obtained analyzing aniline blue-stained intact SPZ (data not shown) suggesting that, in our animal model, head length reflects nuclear length.

3.3. Sperm histone retention analysis after E_2 treatment

Aniline blue staining was used to analyze histone retention in SPZ from Cnr1$^{-/-}$ mice *in vivo* treated with vehicle alone (Control), with E_2 ± ICI or ICI alone in comparison with untreated Cnr1$^{+/+}$ animals, used as physiological control. Results are reported in Table 3 and show that the percentage of white SPZ (no histone retention) was significantly higher in Cnr1$^{+/+}$ than in Control ($P < 0.01$). Conversely, the percentage of dark blue SPZ (high histone retention) was higher in Control than in Cnr1$^{+/+}$ mice ($P < 0.01$). The percentage of light blue SPZ (low histone retention) was slightly higher in Control than in Cnr1$^{+/+}$ mice ($P < 0.01$). This picture close matched data reported in Table 1, thus excluding putative effects mediated by the vehicle. The pharmacological treatment of Cnr1$^{-/-}$ mice with E_2 restored the percentage of white, light and dark blue SPZ to Cnr1$^{+/+}$ values (Cnr1$^{+/+}$ vs E_2 values were not statistically different). ICI treatment counteracted the E_2 effect restoring the Cnr1$^{-/-}$ phenotype, while no effect was observed in the mice group treated with ICI alone.
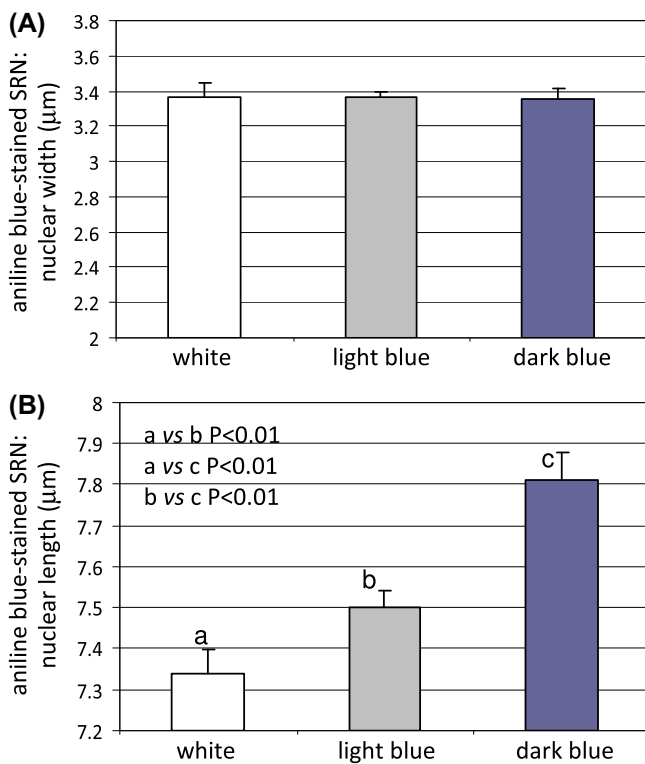

Fig. 3. Nuclear width (A) and length (B) of white, light and dark blue-stained SRN. Results are reported as mean values ± SD. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

condensation and DNA damage are reported in Table 2. As previously reported on SPZ collected from *caput* epididymis (Chioccarelli et al., 2010), HDS (percentage of SPZ with uncondensed chromatin) or DD (percentage of SPZ with DNA damage) values were significantly higher in the Cnr1$^{-/-}$ ($P < 0.01$) as compared with Cnr1$^{+/-}$ or Cnr1$^{+/+}$ which showed matching values of both HDS and DD.

The correlation analysis (Fig. 1) showed a tight and positive relationship between the number of dark blue SPZ (i.e. SPZ highly retaining histones) and those with uncondensed chromatin (panel A; $r = 0.96$) or DNA damage (panel B; $r = 0.96$). No correlation was

3.4. Sperm nuclear size evaluation after E_2 treatments

In vivo treatment. PI staining was used to analyze nuclear size (width and length) of SPZ from $Cnr1^{-/-}$ mice treated with vehicle (Control), with $E_2 \pm$ ICI or ICI alone. SPZ from untreated $Cnr1^{+/+}$ mice were used as physiological control for nuclear size.

Nuclear length was significantly ($P < 0.01$) lower in $Cnr1^{+/+}$ (7.44 ± 0.05 μm) than in $Cnr1^{-/-}$ Control group (7.74 ± 0.06 μm). The *in vivo* treatment with E_2 significantly ($P < 0.01$) decreased nuclear length (7.45 ± 0.06 μm) to $Cnr1^{+/+}$ values (7.44 ± 0.05 μm). This effect was completely counteracted by co-treatment with the ICI (7.75 ± 0.08), while no effect was observed in the mice group treated with ICI alone (Fig. 4A). Conversely, nuclear width of SPZ was not affected by any treatment.

In vitro treatment. Epididymal SPZ samples from $Cnr1^{-/-}$ mice were incubated *in vitro* with vehicle (Control), with $E_2 \pm$ ICI or ICI alone and then stained with PI for nuclear size evaluation. SPZ samples of untreated $Cnr1^{+/+}$ mice were used as physiological control for nuclear size. Results showed that sperm nuclear width and length were not affected by *in vitro* treatments (Fig. 4B) at any E_2 concentration.

3.5. Threshold value for sperm nuclear size

Width and length of white-(open circle) and dark blue-(filled circle) nuclei (i.e. sperm SRN) have been scattered on diagram (Fig. 5) and the values (width + length) were used to calculate the threshold value for "good" sperm nuclear size. The threshold value at the 5th percentile was 9.97 μm and represented a border line (dotted line) useful to discriminate white nuclei (SPZ with mature chromatin, normal histone content and well protamined DNA) from dark blue nuclei (SPZ with immature chromatin, abnormal
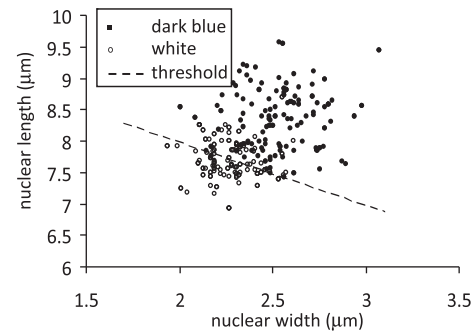

Fig. 5. Nuclear width and length of white-(open circle) and dark blue-(filled circle) stained SRN have been scattered on diagram and used (width + length) to calculate the threshold value for "good" sperm nuclear size.

histone content and poorly protamined DNA). Using this threshold as a test to chose (negative test when width + length < 0.997 μm) or exclude a nuclei (positive test when width + length ⩾ 0.997 μm), in our sample there were 143 true positive nuclei, 2 false negative nuclei, 64 false positive nuclei, 79 true negative nuclei; thus, this test had a sensibility of 0.99, a specificity of 0.55, a positive predictive value of 0.69, a negative predictive value of 0.97.

4. Discussion

We recently characterized the reproductive phenotype of $Cnr1^{-/-}$ mice and reported that males show normal progression of spermatogenesis (Cacciola et al., 2008a), produce SPZ (Cobellis et al., 2010; Ricci et al., 2007) and are fertile although they show high percentage of SPZ with poor chromatin quality (Battista et al.,
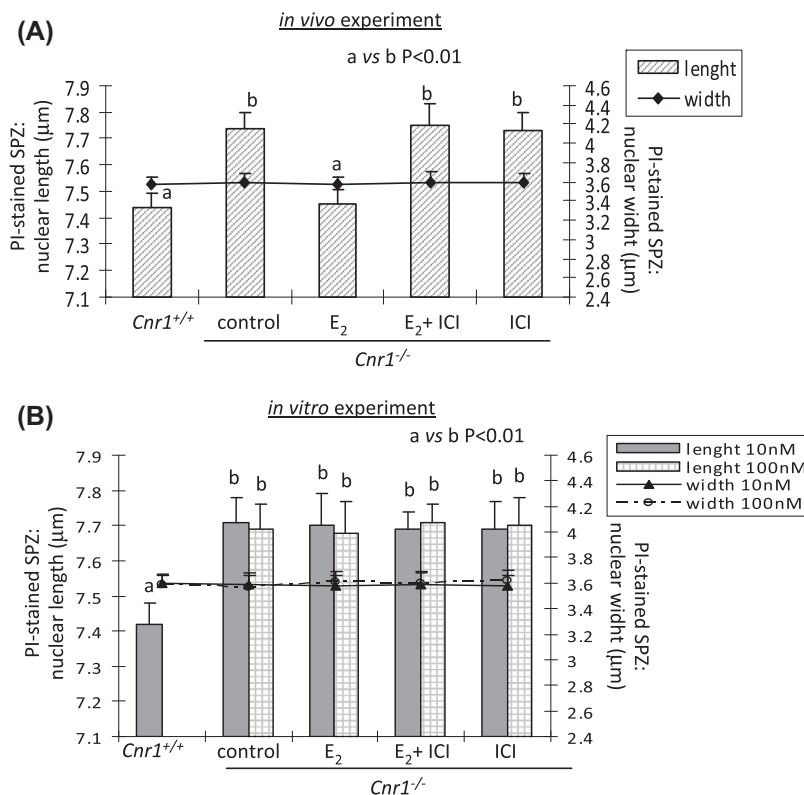

Fig. 4. Nuclear width and length of PI-stained SPZ collected from $Cnr1^{+/+}$, animals, and from $Cnr1^{-/-}$ animals *in vivo* treated with vehicle (Control) or with $E_2 \pm$ ICI (A). Nuclear width and length of PI-stained $Cnr1^{-/-}$ SPZ *in vitro* treated with vehicle (Control) or with $E_2 \pm$ ICI at different doses in comparison with untreated $Cnr1^{+/+}$ SPZ (B). Results are reported as mean values ± SD.

2012; Chioccarelli et al., 2010). Primarily, the genetic inactivation of *Cnr1* affects chromatin remodeling mechanisms that occurs in SPT during spermiogenesis, by reducing histone displacement and so sperm chromatin quality indices (chromatin condensation and DNA integrity). Indeed, in *caput* epididymis from $Cnr1^{-/-}$ animals, the number of SPZ with histone retention as well as the number of SPZ with uncondensed chromatin or with DNA damage was higher than in $Cnr1^{+/+}$ and $Cnr1^{+/-}$ animals (Chioccarelli et al., 2010). Therefore, using epididymal SPZ from $Cnr1^{+/+}$, $Cnr1^{+/-}$ and $Cnr1^{-/-}$ animals, we carried out a correlation analysis between percentage of SPZ with histone retention and those with uncondensed chromatin 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 affects nuclear size of SPZ.

Using a standard optical microscope and staining methods, we analyzed chromatin features (histone retention, chromatin condensation, DNA damage) of epididymal SPZ collected from $Cnr1^{+/+}$, $Cnr1^{+/-}$ and $Cnr1^{-/-}$ animals. We observed that, independently of genotype, SPZ appeared morphologically normal (data not shown) although aniline blue dye reveled a heterogeneous population of SPZ, where three classes of stained SPZ were identified (white/gray, light and dark blue, depending on variable histone content). The percentage values of SPZ differentially stained confirm previous results (Chioccarelli et al., 2010) and show that histone retention was higher in $Cnr1^{-/-}$, significantly lower in $Cnr1^{+/-}$ and further decreased in $Cnr1^{+/+}$ animals. This was revealed by the higher number of dark blue SPZ produced by $Cnr1^{-/-}$ mice (the percentage of light blue SPZ was comparable in $Cnr1^{+/-}$ and $Cnr1^{-/-}$ mice while the percentage of the dark blue SPZ was higher in $Cnr1^{-/-}$ than $Cnr1^{+/-}$). Likewise, HDS (percentage of SPZ with uncondensed chromatin) or DD (percentage of SPZ with DNA damage) values were significantly higher in the $Cnr1^{-/-}$ than in $Cnr1^{+/-}$ or $Cnr1^{+/+}$. Several studies propose that uncondensed chromatin and DNA damage are related to each other and are secondary to disrupted histone displacement (Carrell et al., 2007). In agreement, we found a significant positive correlation between high histone retention (number of dark blue SPZ) and uncondensed chromatin or DNA damage suggesting that abnormal histone retention may be used as index of poor sperm chromatin quality. Interestingly, no correlation was found when light blue SPZ (SPZ with slight histone retention) were used in the correlation analysis (data not shown), suggesting their null or limited contribution to chromatin quality. It is known that histone displacement occurs in elongating and condensing SPT and is responsible for a radical change in chromatin folding useful to generate SPZ with a small, hydrodynamic and compact nucleus (Johnson et al., 2011). Indeed, histone-to-protamine exchange promotes thickening of chromatin fibers, uniform chromatin condensation in an anterior-to-posterior direction and reduction of the nuclear size (Dadoune, 2003). Therefore, we reasoned that histone retention and poor chromatin quality might negatively influence nuclear dimensions of SPZ. As a consequence, we compared nuclear length and width of PI-stained SPZ from $Cnr1^{+/+}$, $Cnr1^{+/-}$ and $Cnr1^{-/-}$ mice. We found that mean values of length was higher in $Cnr1^{-/-}$ than in $Cnr1^{+/+}$ or $Cnr1^{+/-}$ mice, whose values were comparable, and concluded that this correlated with the higher number of dark blue SPZ produced by $Cnr1^{-/-}$ mice. Indeed, nuclear length was higher in dark blue-stained SRN (with high histone retention), significantly lower in the light blue (with slight histone retention) and further decreased in the white/gray (with normal histone retention). This clearly suggests that histone retention causes nuclear size elongation of SPZ. Interestingly, either PI-stained SPZ from $Cnr1^{-/-}$ animals or dark blue-stained SRN were longer, but not larger. This likely happened since histone displacement and chromatin condensation occur in a rostral-to-caudal direction (Dadoune, 2003) and failure of this mechanism produces nuclear swelling along the longitudinal axes.

Estrogens regulates male reproduction (Carreau and Hess, 2010; Chianese et al., 2013; Cobellis et al., 1999, 2002, 2003, 2005a, 2005b, 2008; Gill-Sharma et al., 2011; Pierantoni et al., 2002; Ruwanpura et al., 2010; Staub et al., 2005), and in agreement with studies reporting an interfering effect of estrogens on SPT elongation (Shetty et al., 1997, 1998), we recently demonstrated that estrogens, probably via FSH, affect spermiogenesis and regulate chromatin remodeling of germ cells (Cacciola et al., 2013). In particular, they preserve chromatin condensation and DNA integrity of SPZ by promoting histone displacement in SPT. Indeed, $Cnr1^{-/-}$ males show low levels of circulating estrogens and when treated with E_2 they rescue chromatin quality of SPZ by restoring histone content, chromatin packaging and DNA integrity to untreated $Cnr1^{+/+}$ values. We reasoned that E_2-treated $Cnr1^{-/-}$ mouse was the useful experimental approach (i.e. litmus test) to confirm the relationship between nuclear length and chromatin quality of SPZ. Therefore, we treated $Cnr1^{-/-}$ mice with vehicle or with $E_2 \pm$ ICI. Epididimal SPZ were stained with aniline blue or PI and analyzed for histone retention or nuclear length in comparison to $Cnr1^{+/+}$ SPZ samples. Histone retention/displacement was used 1) as sperm chromatin quality index and 2) as experimental control of E_2 treatment efficiency. The results showed that the percentages of white, light and dark blue SPZ from untreated $Cnr1^{+/+}$ animals were the mirror image of the percentages counted in $Cnr1^{-/-}$ Control group. E_2 treatment, *via* ER, restored the percentages of white, light and dark blue SPZ to $Cnr1^{+/+}$ values thus confirming either rescue of chromatin quality of SPZ (Cacciola et al., 2013) or efficiency of E_2 treatment. Very interestingly, as hypothesized, E_2 was also able to reduce the mean nuclear length of $Cnr1^{-/-}$ SPZ to $Cnr1^{+/+}$ values demonstrating that nuclear size of SPZ is an additional parameter responsive to estrogens since dependent on chromatin quality. To exclude a direct effect of E_2 on SPZ we carried out a further experiment by incubating epididimal $Cnr1^{-/-}$ SPZ samples with vehicle or with $E_2 \pm$ ICI. No change of nuclear size was observed.

Altogether, the present data confirm that mice produce a heterogeneous population of SPZ because of variable histone content (Chioccarelli et al., 2010). Furthermore, we suggest that the small nucleus of white SPZ (containing chromatin that did not retain histones) was fully condensed and able to preserve DNA from damage. On the contrary, the longer nucleus of dark blue SPZ (containing chromatin that retained histones) was uncondensed and unable to preserve DNA from damage. We propose that nuclear dimensions of white and dark blue SPZ can be a good indicator of the chromatin quality of SPZ. Therefore, we have scattered width and length of white and dark blue nuclei (i.e. sperm SRN; we have excluded the light blue since the slight histone retention did not interfere with chromatin quality). The scatter diagram shows a partial overlapping of these two classes of nuclei, thus avoiding a complete separation between the unstained (white nuclei with mature chromatin) and stained ones (dark blue nuclei, with immature chromatine). On this basis, we planned a statistical test with high sensitivity and reasonable specificity, in order to identify a cut-off value excluding 95% of dark blue nuclei. As a consequence we have been able to identify the lower 5th percentile (9.97 μm) in nuclear dimensions (width plus length). This cut-off value (9.97 μm) represents a threshold value, which allows to discriminate with high sensitivity immature dark blue nuclei (i.e. SPZ with histone retention, uncondensed chromatin and DNA damage) from mature white nuclei (i.e. SPZ with protamined and condensed chromatin, and safe DNA).

This study extend previous observations (Chioccarelli et al., 2010) and shows that histone retention directly correlates either with uncondensed chromatin or DNA damage and produces SPZ with greater nuclear length. Interestingly, the nuclear length appears to be an E_2-responsive morphological parameter which is

related to chromatin quality. Furthermore, it may be used as useful tool to discriminate, among SPZ appearing morphologically normal, those with good chromatin quality. Interestingly, in ART field, abnormal nuclear size of SPZ is commonly considered to be of poor prognosis (Bartoov et al., 2002). More interestingly, a recent article describes a tight correlation between semen analysis by motile sperm organelle morphology examination and DNA damage (Oliveira et al., 2010).

In conclusion our results, if adequately shifted to the andrological field, may help to easy select SPZ with good chromatin quality, in order to carry out an *in vitro* fertilization by intracytoplasmic sperm injection.

Disclosure Statement

The authors have nothing to disclose.

Acknowledgments

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