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Spermatozoa from mice deficient in Niemann-Pick disease type C2 (NPC2) protein have defective cholesterol content and reduced *in vitro* fertilising ability

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Abstract. The cholesterol content of the sperm membrane is regulated during both maturation in the epididymis and capacitation in the female tract, two processes required for the spermatozoa to acquire their fertilising ability. Because Niemann-Pick disease, type C2 (NPC2) protein is one of the most abundant components of the epididymal fluid and contains a functional cholesterol-binding site that can transfer cholesterol between membranes, it has been suggested for years that NPC2 could be involved in the regulation of cholesterol levels in spermatozoa during epididymal maturation. In the present study, western blot and immunohistochemistry analyses demonstrated significant levels of NPC2 in the mouse epididymal epithelium. Epididymal spermatozoa obtained from *NPC2*$^{-/-}$ mice were morphologically normal and had normal motility parameters, but had a reduced cholesterol content compared with that of wild-type (WT) spermatozoa, as determined by both biochemical and by flow cytometry analyses. These results suggest that NPC2 could be involved in regulating cholesterol levels in spermatozoa during epididymal maturation. To understand the relevance of epididymal NPC2 for sperm function, the ability of spermatozoa to undergo events influenced by epididymal maturation, such as capacitation and fertilisation, were compared between WT and *NPC2*$^{-/-}$ mice. Capacitated *NPC2*$^{-/-}$ spermatozoa exhibited defective tyrosine phosphorylation patterns and a reduced ability to fertilise cumulus–oocyte complexes compared with WT spermatozoa, supporting the relevance of mouse epididymal NPC2 for male fertility.

Additional keywords: epididymis, fertilisation.

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Introduction

The migration of spermatozoa along the epididymis involves changes at both structural and biochemical levels (Cornwall 2009). This epididymal maturation involves the remodelling of sperm surface components (i.e. protein processing, relocalisation and addition to the sperm surface; Dacheux *et al.* 2003; Aitken *et al.* 2007) and changes in the composition and distribution of lipids, in particular phospholipids and sterols (Parks and Hammerstedt 1985; Haidl and Opper 1997; Rejraji *et al.* 2006). It has been proposed that redistribution of molecules in the plasma membrane during epididymal maturation allows the formation of new signalling complexes that are kept inactive during storage in the cauda epididymis but are relevant for spermatozoa to express their fertilisation potential in the female tract (Jones 1998; Cornwall 2009). As in most cell types, cholesterol participates in the regulation of plasma membrane fluidity and permeability in spermatozoa, as well as in the motility of proteins and receptors (Hall *et al.* 1991; Haidl and Opper 1997). The total amount of sperm cholesterol and the ratio of cholesterol to phospholipids changes during epididymal maturation; these changes are highly species specific, with cholesterol content and the cholesterol : phospholipid ratio decreasing

in some species but increasing in others (Parks and Hammerstedt 1985; Hall *et al.* 1991; Haidl and Opper 1997; Rejraji *et al.* 2006). Despite these differences, the amount and distribution of cholesterol seems to be important for sperm function in mammals, because the plasma membrane of mammalian spermatozoa shows abundant cholesterol-enriched lipid 'rafts', specialised membrane microdomains that serve as organising centres for the assembly of signalling molecules (Travis *et al.* 2001; Cross 2004; Shadan *et al.* 2004).

During transit along the female tract, mature spermatozoa undergo additional modifications before they are fully competent to fertilise eggs in a process known as capacitation (Yanagimachi 1994). In most species, cholesterol efflux from the plasma membrane is one of the early events closely associated with sperm capacitation and it seems to be a requirement to undergo the acrosome reaction (AR; Cross 1998; Ramalho-Santos *et al.* 2002). Cholesterol release induces lipid rafts to migrate and cluster over the anterior sperm head and this new molecular distribution activates downstream signalling pathways (Cross 1998; Jones *et al.* 2007; Gadella *et al.* 2008). The first and best described molecular cascade related to capacitation is that involving the activation of protein kinase A (PKA), which results in the phosphorylation of specific tyrosine residues in sperm proteins (Visconti *et al.* 1995). Activation of PKA during capacitation has been shown to be essential for gamete fertilisation because it is required for other sperm functions, such as hyperactivated motility (Tash and Means 1983), zona pellucida binding (Naz 1996; Tomes *et al.* 1996; Liu *et al.* 2006) AR (Naz 1996) and egg binding and fusion (Urner *et al.* 2001). Interestingly, cholesterol release from the plasma membrane of mature spermatozoa during capacitation has been shown to induce sperm tyrosine phosphorylation (Visconti *et al.* 1999*a*, 1999*b*). Despite the relevance of cholesterol for sperm maturation, capacitation and fertilisation, the molecular mechanisms governing its levels and distribution are far from being completely understood. Cholesterol acceptors, such as apolipoproteins (Apo) ApoA-I and ApoJ, have been shown to be secreted from epithelial cells of the epididymis (Law *et al.* 1997), uterus and oviduct (Argraves and Morales 2004). It has been suggested that apolipoproteins in the epidymal fluid associate with specific ATP-binding cassette, sub-family A, member 1 (ABCA1) receptors localised in the sperm plasma membrane that could serve as cholesterol acceptors (Morales *et al.* 2008).

Human epididymis 1 (HE1) protein was originally identified as one of the most abundant proteins present in human epididymal fluid (Kirchhoff *et al.* 1996) and was later shown to be a cholesterol-binding protein in porcine spermatozoa (Okamura *et al.* 1999). HE1 turned out to be identical to Niemann-Pick disease, type C2 (NPC2) protein (Naureckiene *et al.* 2000), encoded by one of the genes mutated in patients with Niemann-Pick type C (NPC) disease. In addition to its localisation in the epididymal fluid and other secretions, NPC2 is found in lysosomes and has been shown to be relevant for the efflux of cholesterol from this organelle (Infante *et al.* 2008). For this reason, NPC patients have progressive lipidosis that results in the gradual deterioration of the central nervous system, visceral symptoms and premature death (Pentchev *et al.* 1997). Structure–function analyses of NPC2 demonstrated that the protein contains a functional cholesterol-binding site and can transfer cholesterol between membranes (Friedland *et al.* 2003; Babalola *et al.* 2007; Infante *et al.* 2008; Kwon *et al.* 2009).

Epididymal NPC2 was first related to male fertility when its expression was shown to be downregulated in vasectomised men (Legaré *et al.* 2004). A later study showed that after religation of the vas deferens (vasovasostomy), a subpopulation of ejaculated spermatozoa showed high association of NPC2, increased cholesterol content and reduced motility, leading the authors to propose an interesting hypothesis whereby NPC2 in the epididymal fluid participates in cholesterol efflux from spermatozoa during epididymal sperm maturation (Légaré *et al.* 2006). More recently, the same group showed that incubation of bovine spermatozoa with purified NPC2 lowered sperm cholesterol content and disorganised lipid rafts (Girouard *et al.* 2008).

NPC2-deficient ($NPC2^{-/-}$) mice were produced years ago as a rodent model for human NPC disease (Sleat *et al.* 2004). Due to aberrant homologous recombination during targeting of the *NPC2* gene, $NPC2^{-/-}$ mice do not lack NPC2 completely and instead are hypomorphs expressing 0–4% NPC2 protein in different tissues. $NPC2^{-/-}$ mice grow normally until 55 days of age, when the first signs of neurodegeneration occur: tremor, defective movements and weight loss. We have shown previously that female $NPC2^{-/-}$ mice are infertile due to anovulation and defective steroidogenesis (Busso *et al.* 2010). Male $NPC2^{-/-}$ are also infertile because they do not mate (D. Busso, pers. obs.), probably due to the locomotor dysfunction that begins shortly before they reach sexual maturity.

In the present study we analysed the presence and localisation of NPC2 protein in the epididymis and compared different qualitative and functional parameters between epididymal spermatozoa from $NPC2^{-/-}$ and wild-type (WT) mice.

Materials and methods

Mice

The $NPC2^{-/-}$ BALB/c mice used in the present study were developed by Dr Peter Lobel (Sleat *et al.* 2004). We used mice between 55 and 65 days of age to ensure reproductive maturity and to avoid the detrimental effects observed in $NPC2^{-/-}$ mice. Whenever possible, different parameters were compared in WT and $NPC2^{-/-}$ mice from the same litter. When this was not possible, age-matched WT mice were used. In protocols requiring post-surgical survival, mice were anaesthetised with an intraperitoneal injection of a mixture of 80–100 mg kg^{-1} ketamine and 5–10 mg kg^{-1} xylazine; in experiments involving sperm retrieval, mice were killed by cervical dislocation to ensure good viability of the recovered cells. C57BL/6J mice where the $NPC2^{-/-}$ mutation had been back-crossed for at least seven generations were used for IVF assays and sperm motility. The experimental protocols adhered to the National Research Council (NRC) publication *Guide for Care and Use of Laboratory Animals* (Committee for the Update of the Guide for the Care and Use of Laboratory Animals; National Research Council 2011). The studies were approved by the Ethics Committee for Animal Welfare of the Faculty of Medicine of the Pontificia Universidad Católica de Chile.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis and western blot determination of NPC2

Caput, corpus and cauda section of the epididymis were homogenised in ice-cold 50 mM Tris–HCl buffer (pH 7.4) containing 1% Nonidet P-40 (Sigma Chemical, St Louis, MO, USA), 0.2 mM phenylmethylsulfonyl fluoride (PMSF; Sigma Chemical) and protease inhibitor cocktail, which was used in a 1 : 50 dilution according to the manufacturer's instructions (Sigma Chemical). Samples were separated by 15% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions according to the method of Laemmli (1970), and proteins were electrotransferred to nitrocellulose membranes (Towbin *et al.* 1979). Membranes were blocked for 1 h with powdered skim milk (2% in phosphate-buffered saline (PBS)) before being incubated overnight at 4°C with anti-NPC2 antibodies (diluted 1 : 750; Atlas Antibodies, AlbaNova University Center, Stockholm, Sweden), anti-β-actin antibodies (diluted 1 : 5000; Sigma Chemical) or anti-α tubulin antibodies (diluted 1 : 8000; Sigma Chemical). The membranes were then washed thoroughly with PBS containing 0.1% Tween and incubated for 1 h with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies. After washing as indicated previously, the immunoreactive proteins were detected by an enhanced chemiluminescence western blotting kit (Perkin Elmer, Waltham, MA, USA). All incubations were performed at room temperature.

Histology and immunohistochemistry

Epididymides and testes were placed in Bouin fixative for at least 12 h, dehydrated and embedded in paraffin. Sections (8 μm) were cut, mounted on slides and kept at 4°C until use. Slides were rehydrated and stained with periodic acid-Schiff and Harris haematoxylin solution (Sigma Chemical) for histological analysis. For immunohistology, rehydrated sections were incubated for 1 h in PBS containing 1% bovine serum albumin (PBS-BSA) and then exposed overnight in a humidified chamber to anti-NPC2 at a 1 : 750 dilution in PBS-BSA. Normal rabbit IgG (Sigma Chemical) at the same IgG concentration was used as a negative control. After washing in PBS, the slides were developed using the anti-rabbit–HRP–diaminobencidine ultravision detection system (LabVision, Fremont, CA, USA). Finally, sections were counterstained with haematoxylin. Slides were dehydrated and mounted with Entellan New Mounting Medium (Merck, Damstadt, Germany). Slides were examined and photographed using a Zeiss microscope (Carl Zeiss, Thornwood, NY, USA) and a Nikon Coolpix 4500 camera (Nikon, Tokyo, Japan).

Analysis of sperm number, viability and morphology

Mouse cauda sections were separated from the epididymides and placed in 0.3 mL PBS at 37°C under mineral oil. Three incisions were made with fine scissors and the PBS drops containing the tissue were incubated for 30 min to allow the spermatozoa to swim out. Aliquots of freshly obtained spermatozoa were used to analyse sperm number, viability and morphology by visualisation under a light microscope. One hundred spermatozoa were counted for each treatment, and at least three independent experiments were performed. For viability assessment, sperm suspensions were incubated for 5 min in 0.2% eosin prior to visualisation and cells excluding the dye were scored as live spermatozoa. For morphological analysis, aliquots of spermatozoa were fixed in 4% paraformaldehyde (PFA) in PBS and stained with Coomasie brilliant blue prior to visualisation.

In vitro *sperm capacitation*

Mouse cauda epididymal spermatozoa were allowed to disperse in 0.3 mL M2 (Sigma Chemical) or Whittingham capacitation medium (Biggers *et al.* 1971) for 15–30 min and the final concentration of spermatozoa was determined using a Neubauer chamber. Aliquots of the original suspension were diluted to $2–5 \times 10^6$ cells mL^{-1} in 0.5 mL fresh capacitation medium supplemented with 0.3% BSA and incubated for up to 120 min at 37°C in an atmosphere of 5% CO_2 in air.

Determination of sperm cholesterol content

Folch method

Spermatozoa obtained in PBS at 37°C were homogenised and incubated with 6 volumes of chloroform–methanol (Merck) in a ratio of 2 : 1 to extract total cholesterol according to the method of Folch *et al.* (1957). Radioactive cholesterol (0.01 μCi) was added to each sample as an internal standard for cholesterol extraction. Samples were incubated at 50°C for 1 h and distilled water was added up to 10 mL before incubating the tubes at 4°C overnight. Samples were then centrifuged at 900*g* at 4°C for 20 min in a Sorvall centrifuge (Model RT 6000B; Thermo Fisher, Waltham, MA, USA) and the lower phase was collected and evaporated under nitrogen. Cholesterol was estimated using an internal standard by an enzymatic and colorimetric procedure described previously (Allain *et al.* 1974). Before evaporation of the lower phase radioactivity containing cholesterol, radioactivity was determined in 200 μL aliquots (in duplicate) to calculate extraction efficiencies. Results are expressed as mg cholesterol per 10^7 spermatozoa.

Filipin staining

The cholesterol content in the sperm plasma membrane was evaluated by filipin binding to live spermatozoa and visualisation by fluorescence, as reported previously (Takeo *et al.* 2008; filipin excitation wavelength 365 nm; emission wavelength 420 nm). Spermatozoa were incubated in 500 μL PBS containing 25 μg mL^{-1} filipin (Sigma Chemical) for 30 min in the dark and washed by centrifugation for 10 min at 5000*g*. Aliquots (50 μL) of filipin-stained spermatozoa were fixed with 4% PFA, mounted on glass slides using Fluoromount-G anti-fade medium (Southern Biotech, Birmingham, AL, USA) and observed under an epifluorescence microscope (BX51; Olympus, Hopkinton, MA, USA). The remaining sperm suspensions were used for quantification of cholesterol using filipin fluorescence by flow cytometry using a cell sorter (FACSaria III; BD, Franklin Lakes, NJ, USA) with a 375 nm : >7 mW top hat shape near the ultraviolet laser diode and a 450/20 bandpass filter, and excitation and emission wavelengths of 365 and 420 nm, respectively. Dead cells (i.e. cells that were positive for the DNA-binding probe propidium iodide (PI)) were excluded from analysis. For

PI staining, a 561 nm : >50 mW elliptical shape laser diode and a 610/20 nm bandpass filter (excitation 535 nm; emission 617 nm) were used. Live cells were separated on the basis of forward and side scatter distribution. The main population corresponding to that concentrating the highest number of events was identified as the sperm population and selected for analysis. To eliminate the contribution of debris within the selected population to the filipin fluorescence detected, filipin staining was performed in combination with live and dead staining (LIVE/DEAD sperm viability kit; Molecular Probes, Eugene, OR, USA). This procedure uses PI staining in combination with the nuclear stain SYBR14 and allows identification of debris ($SYBR14^-$) versus nucleated spermatozoa ($SYBR14^+$) in the PI^- sample. Spermatozoa incubated in PBS alone were used as a negative control for autofluorescence in both microscopic and FACS analyses. Capacitated spermatozoa and spermatozoa incubated with 1.5 mM methyl-β-cyclodextrin (MBCD) were included as internal controls in these experiments because after these treatments, the cholesterol content in the plasma membrane is expected to be reduced.

Evaluation of the AR

The occurrence of the AR was evaluated by Coomassie brilliant blue (CBB) staining. Spermatozoa were fixed in 1 volume of 8% PFA in PBS for 1 h at 4°C, washed with 0.1 M ammonium acetate (pH 9) by centrifugation at room temperature at 3000*g* for 10 min, mounted on slides and air dried. Slides were washed by successive immersions in water, methanol and water (5 min each) and then incubated in CBB solution (0.22% CBB in 50% methanol and 10% acetic acid). After staining, slides were washed thoroughly with distilled water, mounted and observed immediately to avoid diffusion of the stain. Spermatozoa were scored as 'acrosome intact' when a bright blue staining was observed on the dorsal region of the acrosome or as 'acrosome reacted' when staining was either patchy or absent. One hundred spermatozoa were counted in five independent experiments.

Determination of phosphorylation in sperm tyrosine residues

After incubation in capacitating medium for 30, 60, 90 and 120 min, spermatozoa were collected and washed once by centrifugation at 5000*g* with 0.5 mL of 0.5M Tris–HCl (pH 6.8) supplemented with 1 mM ortovanadate and 1 mM PMSF at 4°C. Samples were resuspended in Laemmli sample buffer, vortexed and boiled for 5 min. Sperm protein extracts (corresponding to $1–2 \times 10^6$ spermatozoa per lane) were separated by 7.5% SDS-PAGE and transferred onto nitrocellulose membranes, which were then probed with anti-phosphotyrosine monoclonal antibody (1 : 2000; clone 4G10; Upstate, Lake Placid, NY, USA) as described previously (Da Ros *et al.* 2004*a*).

Analysis of sperm motility

Sperm motility was analysed at the Centro de Espermiogramas Digitales Asistidos por Internet (CEDAI; http://www.cedai.cl, accessed June 2012) based on analytical computational tools developed in SCIAN-Lab (http://www.scian.cl, accessed June 2012) on the basis of Interactive Data Language (IDL) 8.2 (ITT Corporation, Bolder, CO, USA). Image acquisition (*xy* resolution 780/580 pixels, 650/484 μm, 8-bit intensity scale) was performed at 30 Hz with a charge-coupled device (CCD) scA780-54 gc camera (Basler, Ahrensburg, Germany) with a light-emitting diode (LED)-illuminated Zeiss microscope (Axio Lab.A1; ×10 objective; Ph1). Sperm samples (20 μL) were pipetted onto Leja motility chambers (20 μm depth; Leja Products, Nieuw Vennep, The Netherlands) to examine microscopic acquisitions. At least three samples from each animal and each time point were analysed following the same procedure. Interactive computational tools for sperm head segmentation, tail segmentation and the tracking of sperm heads were adapted from a system applied for human sperm motility analysis at CEDAI. The quality of the automated sperm tracking was controlled visually to ensure that all sperm trajectories were adequately identified for further quantification. From the sperm trajectories, immotile, slow, progressive and hyperactive spermatozoa were classified through a combination average path velocity (VAP; $\mu m\,s^{-1}$), straight line velocity (VSL; $\mu m\,s^{-1}$), curvilinear velocity (VCL; $\mu m\,s^{-1}$) and the amplitude of lateral head displacement (ALH; μm) between the curvilinear and the average path as follows: immotile spermatozoa, VSL ≤ $13\,\mu m\,s^{-1}$; slow spermatozoa, $13\,\mu m\,s^{-1}$ < VSL ≤ $30\,\mu m\,s^{-1}$ or VCL ≤ $50\,\mu m\,s^{-1}$; progressive spermatozoa, VSL > $30\,\mu m\,s^{-1}$ or $50\,\mu m\,s^{-1}$ < VCL < $100\,\mu m\,s^{-1}$; and hyperactive spermatozoa, ALH ≥ 4 μm, VCL ≥ $100\,\mu m\,s^{-1}$, VSL/VCL < 0.4.

Recovery and treatment of mouse oocytes

Female C57BL/6J mice were superovulated by intraperitoneal injection of 5 IU equine chorionic gonadotropin (Sigma Chemical), followed 48–50 h later by the administration of 5 IU, i.p., human chorionic gonadotropin (hCG; Sigma Chemical). Eggs were obtained from the oviducts of superovulated animals 12–15 h after hCG administration. Cumulus cells were removed by incubating the cumulus–oocyte complexes (COCs) for 3 min in capacitating medium containing 0.1% hyaluronidase (Type IV; Sigma Chemical). For the removal of the zona pellucida (ZP), eggs were treated with acid Tyrode's solution, pH 2.5, for 10-20 s (Nicolson *et al.* 1975).

In vitro *fertilisation*

In these experiments, C57BL/6J *NPC2*$^{-/-}$ adult (55–65 days old) male mice were used in parallel with WT littermates (or age-matched mice). Two mice of each genotype were used in each experiment. Spermatozoa were retrieved by puncture of the cauda epididymides and vas deferens of mice in Human Tubal Fluid Medium (HTF) medium (Quinn *et al.* 1985) and incubation for 15–20 min. Spermatozoa were quantified in a Neubaer chamber and the same number of spermatozoa from WT and *NPC2*$^{-/-}$ mice were used for the insemination of eggs. For IVF assays involving COCs, spermatozoa were diluted in 200 μL drops of HTF medium to a final concentration of $1–2 \times 10^6$ cells mL^{-1} before the addition of the COCs. For gamete fusion assays, spermatozoa were capacitated for 1 h at a concentration of $5–10 \times 10^5$ spermatozoa mL^{-1} and diluted 1 : 10 before insemination of ZP-free eggs. In both cases, gametes were coincubated overnight at 37°C and 5% CO_2 and fertilisation

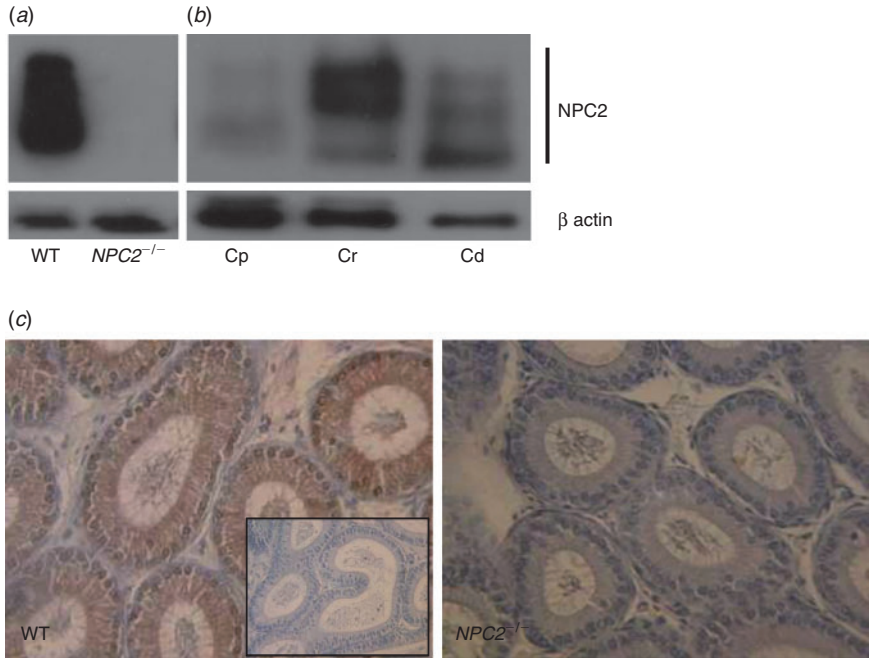


Fig. 1. NPC2 immunolocalisation in the epididymis. Western blot analysis using anti-NPC2 on protein extracts from (*a*) WT and *NPC2*$^{-/-}$ mice, and (*b*) caput (Cp), corpus (Cr) and cauda (Cd) sections of WT epididymis. β actin was used as a loading control in western blots. (*c*) Immunohistochemistry using anti-NPC2 on sections of corpus epididymides from WT and *NPC2*$^{-/-}$ mice. Normal rabbit IgG was used as a primary antibody in the negative control shown in the inset (left panel). Representative results of 3 repetitions are shown.

was determined the next day by visualisation of 2-cell embryos in the fertilisation dishes. A parthenogenesis control was included in both cases, in which COC or ZP-free oocytes were incubated overnight in the absence of spermatozoa.

Statistical analyses

Data are given as the mean ± s.e.m. Differences between values were evaluated by Student's *t*-tests using GraphPad 5.00 (GraphPad Software, La Jolla, CA, USA). In all cases, $P < 0.05$ was considered significant.

Results

NPC2 expression in the epididymis

Western blot analysis was used to evaluate the presence of NPC2 in protein extracts from mouse epididymides. As reported previously (Sleat *et al.* 2004), epididymal NPC2 was detected as a broad band of approximately 20 kDa, given by the superposition of bands in the 15–22 kDa range (Fig. 1*a*). No immunoreactivity was observed in protein extracts of epididymides from *NPC2*$^{-/-}$ mice, confirming the specificity of the antibody. Different bands ranging from 15 to 22 kDa were observed in the caput, corpus and cauda epididymis, with different relative intensities (Fig. 1*b*).

Immunohistochemical studies were performed to localise NPC2 in epididymal tissue sections from WT mice using rabbit anti-NPC2 antibody. Tissue sections treated in the absence of primary antibody or with normal rabbit purified IgGs were used as negative controls. Specific staining was observed in cells of the epididymal epithelium and in spermatozoa within the lumen in the caput, corpus and cauda sections. The results obtained using corpus sections are shown in Fig. 1*c*. Cells in the interstice were unstained in all sections. As expected, no staining was observed in sections of epididymides from *NPC2*$^{-/-}$ mice.

Characterisation of epididymides and spermatozoa in NPC2$^{-/-}$ *mice*

Tissues from male *NPC2*$^{-/-}$ mice and their WT littermates were obtained at 55–70 days of age, corresponding to an age when the mice were sexually mature but had not yet developed severe neurological symptoms (Sleat *et al.* 2004). Neither the testes nor epididymides from *NPC2*$^{-/-}$ male mice showed gross anatomical (Fig. 2*a*, *b*) or histological (Fig. 2*c*) abnormalities. Although the net weight of the epididymides was significantly reduced in *NPC2*$^{-/-}$ compared with WT mice (18.4 ± 4.6 vs 25.4 ± 4.2 mg, respectively; $P < 0.05$; $n \geq 3$), the organ/body-weight ratio was normalised due to the smaller size of *NPC2*$^{-/-}$ adult mice (Sleat *et al.* 2004). The number of spermatozoa obtained per epididymis was reduced in *NPC2*$^{-/-}$ mice, but did not differ significantly from that for WT mice ($2.5 \pm 0.8 \times 10^7$ vs $1.7 \pm 0.5 \times 10^7$ sperm mL^{-1}, respectively; $P = 0.5$; $n = 3$). The *NPC2* deficiency did not affect the viability of spermatozoa in PBS, as evaluated by eosin exclusion ($45 \pm 5\%$ vs $49 \pm 5\%$ in *NPC2*$^{-/-}$ vs WT groups, respectively). No significant differences were found in the proportion of *NPC2*$^{-/-}$ versus WT

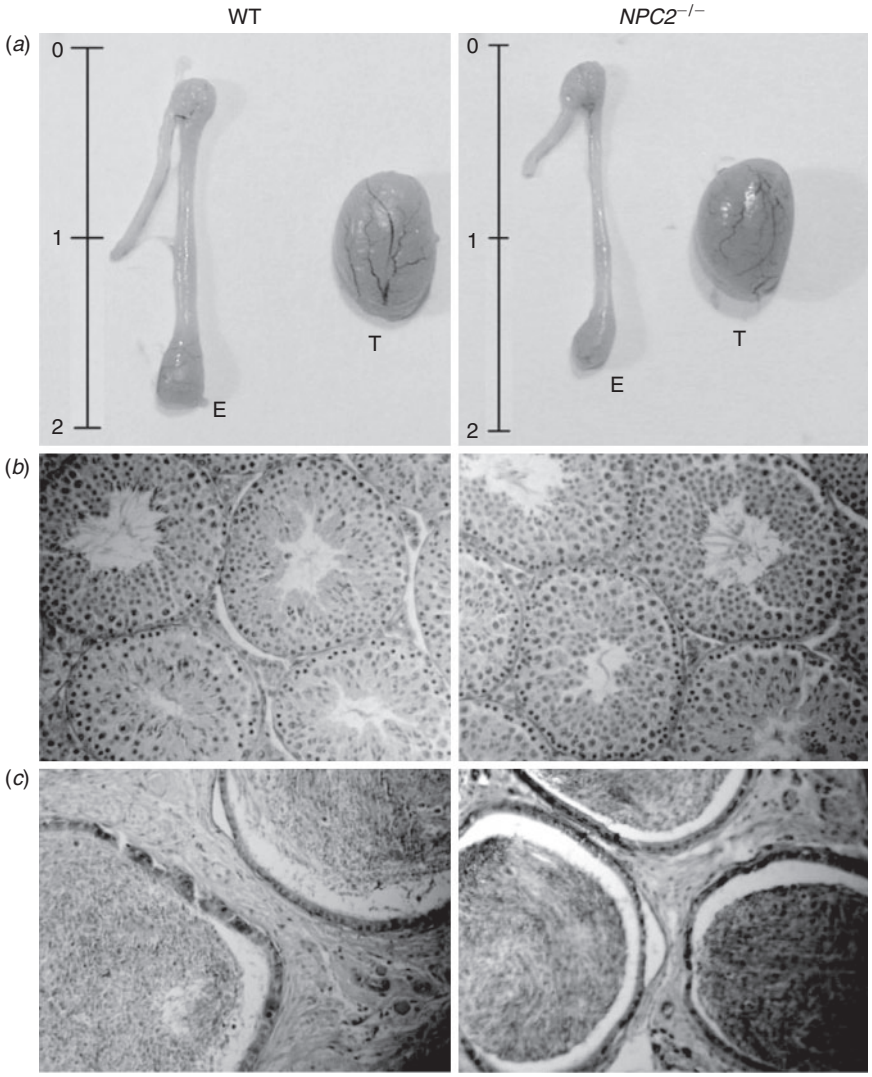


Fig. 2. Analyses of testicles and epididymides from WT and *NPC2*$^{-/-}$ mice. (*a*) Anatomical analysis of epididymides (E) and testes (T). Bars: 0.5 cm. (*b*, *c*) Microphotographs of histological sections from cauda epididymides and testes, respectively. Magnification 200×.

spermatozoa exhibiting different morphological abnormalities, such as blunt heads (10.8 ± 5.6% vs 6.8 ± 3.3%, respectively) or hairpin necks (16.8 ± 6.6% vs 11.6 ± 3.3%, respectively) or in the percentage of spermatozoa with cytoplasmic droplets (between 10% and 15% in all samples analysed, regardless of genotype). In both genotypes, the percentage of normal spermatozoa (73 ± 8% vs 80 ± 4% in *NPC2*$^{-/-}$ and WT groups, respectively; $n = 5$) was comparable to that reported previously for BALB/c spermatozoa (Kawai *et al.* 2006).

Cholesterol content in spermatozoa from NPC2$^{-/-}$ *mice*

Previous results in the human and bovine suggest that NPC2 participates in the efflux of cholesterol from the sperm plasma membrane during epididymal maturation (Légaré *et al.* 2006; Girouard *et al.* 2008). Based on this evidence, our hypothesis was that spermatozoa isolated from the cauda epididymis of *NPC2*$^{-/-}$ mice would have more cholesterol than their WT littermates. However, the total cholesterol content was significantly lower in spermatozoa from *NPC2*$^{-/-}$ compared with WT mice (40% decrease), as determined using the Folch method (Fig. 3*b*). In an alternative method to determine sperm cholesterol levels, live spermatozoa were stained with filipin, a fluorescent indicator of free cholesterol that has been used previously in mouse spermatozoa (Takeo *et al.* 2008). The filipin-stained sperm suspension was split into two aliquots: one was observed under a fluorescent microscope and the other, containing 1×10^6 cells, was analysed by flow cytometry. To analyse spermatozoa with intact plasma membranes, dead or damaged spermatozoa binding the DNA dye PI (32 ± 3% and 29 ± 4% in the WT and *NPC2*$^{-/-}$ groups, respectively) were excluded from the analysis, although their filipin fluorescence was negligible (see Fig. S1 available as Supplementary Material

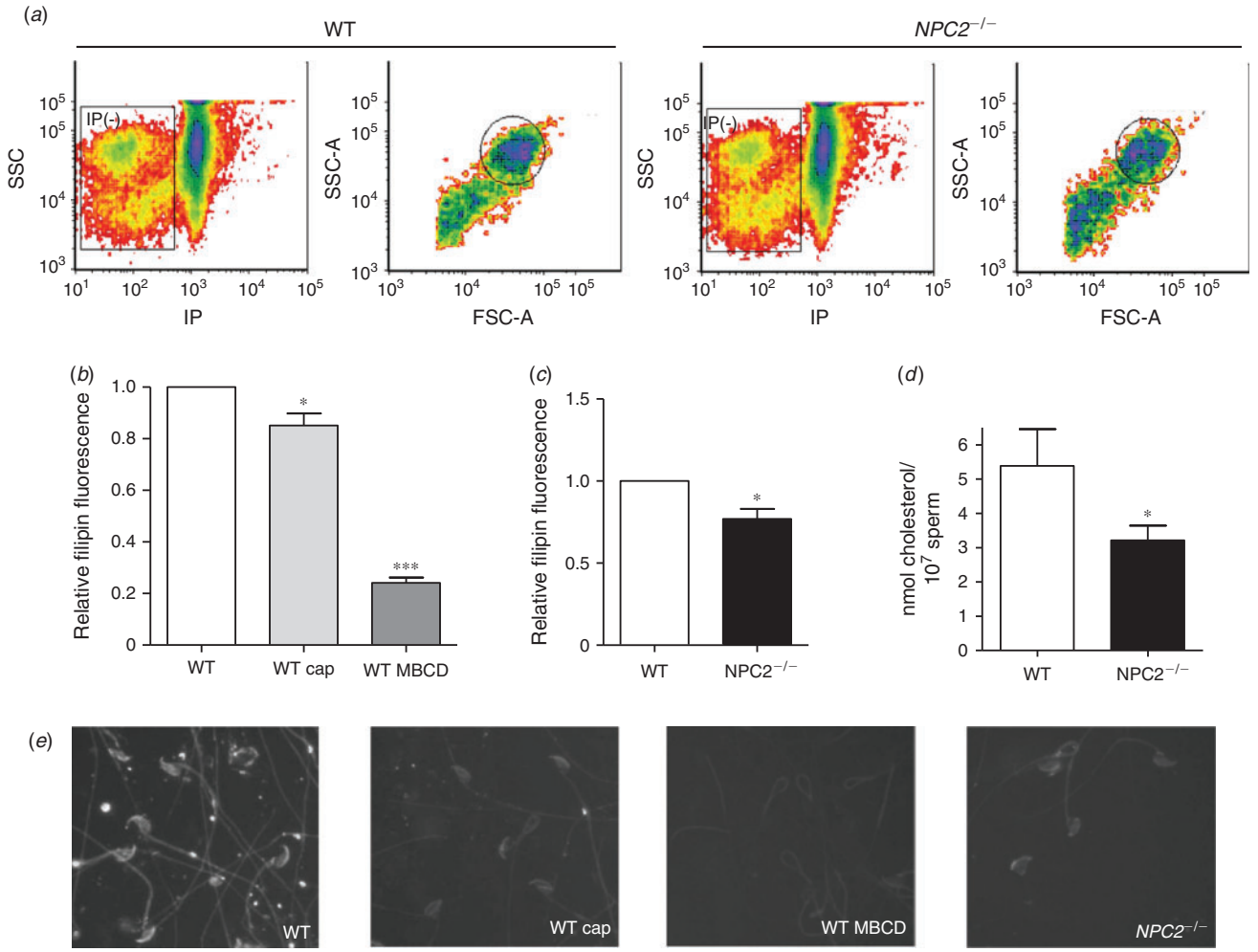


Fig. 3. Determination of sperm cholesterol content in WT and *NPC2*$^{-/-}$ mice sperm. (*a*) Selection of live sperm from cauda epididymides from WT and *NPC2*$^{-/-}$ mice previous to analysis of filipin fluorescence by flow cytometry. Left panels: gating of PI (-) events; Right panel: gating of main population after light scatter analysis. (*b*) Set up of filipin fluorescence quantification using WT fresh (WT) and capacitated sperm (WT cap) and WT sperm previously treated with the cholesterol-removing compound MBCD (WT MBCD). (*c*) Quantification of cholesterol by filipin fluorescence in WT and *NPC2*$^{-/-}$ mice sperm ($n = 4$). (*d*) Quantification of cholesterol by the Folch method (2 independent experiments using 4 pools of sperm from *NPC2*$^{-/-}$ and WT mice). (*e*) Representative pictures of sperm smears after filipin staining. *$P < 0.05$, ***$P < 0.005$.

to this paper). The main PI-negative population was gated for further studies (Fig. 3*a*). Filipin fluorescence flow cytometry analysis was set up using WT spermatozoa under three conditions: fresh, capacitated and incubated with the cholesterol-removing agent MBCD. As expected, capacitated and MBCD-treated spermatozoa had reduced cholesterol as compared with fresh spermatozoa (Fig. 3*c*). Filipin fluorescence was lower in spermatozoa from *NPC2*$^{-/-}$ than WT mice, as determined both by microscopy and FACS analysis (Fig. 3*d*, *e*), confirming the results obtained in the biochemical analysis of total cholesterol content in spermatozoa.

Recent publications by Petrunkina *et al.* have demonstrated that the identification of live spermatozoa on the basis of light scatter analyses and PI selection can lead to an underestimation of non-sperm particles (Petrunkina and Harrison 2010; Petrunkina *et al.* 2010). To eliminate the possible contribution of debris within the selected population to filipin fluorescence, filipin staining was performed in combination with PI and SYBR14 staining. These results revealed that although a significant percentage of particles (~25%) within the PI$^-$ population were SYBR14$^-$, corresponding to non-sperm particles, the contribution of this population to fluorescence was negligible and the reduction in filipin fluorescence was still observed in spermatozoa from *NPC2*$^{-/-}$ mice after gating exclusively for PI$^-$/SYBR14$^+$ events (see Fig. S2).

Functional parameters in capacitated spermatozoa from NPC2$^{-/-}$ *mice*

We hypothesised that the low cholesterol content in spermatozoa from *NPC2*$^{-/-}$ mice may alter the signalling cascades that

are normally activated by the gradual cholesterol efflux that occurs at the sperm plasma membrane during capacitation in the female tract. Supporting this idea, cholesterol staining for filipin remained unchanged in capacitated spermatozoa from *NPC2*$^{-/-}$ mice, but decreased significantly in capacitated spermatozoa from WT mice (Fig. 4*a*), suggesting a defect in the flux of cholesterol response induced by capacitation in spermatozoa from NPC2-deficient mice.

Next, spermatozoa from WT and *NPC2*$^{-/-}$ mice were compared for their ability to undergo phosphorylation in tyrosine residues, a functional parameter related to the process of capacitation and the acquisition of sperm fertilising ability. Spermatozoa incubated *in vitro* under capacitating conditions for different periods of time were subjected to western blotting using an anti-phosphotyrosine antibody (Da Ros *et al.* 2004*a*). The phosphorylation pattern of tyrosine residues in spermatozoa from *NPC2*$^{-/-}$ mice was deficient with respect to that detected in WT spermatozoa in all the time points evaluated (Fig. 4*b*). The occurrence of a spontaneous AR as an endpoint of capacitation was also evaluated. Epididymal spermatozoa from WT and *NPC2*$^{-/-}$ mice were incubated *in vitro* under capacitating conditions and the percentage AR was determined after different incubation times. The kinetics of the AR were overall normal in spermatozoa from *NPC2*$^{-/-}$ mice despite the fact that a non-significant increase in the percentage acrosome-reacted spermatozoa was observed in *NPC2*$^{-/-}$ mice at 60 min (Fig. 4*c*).

Sperm motility evaluated by image processing algorithms allowed the classification of sperm motility into different categories according to the lineality, straight line and curvilinear velocities and amplitude of lateral head displacement. Representative tracks for spermatozoa belonging to the different categories are shown in Fig. 5*a*. Hyperactivated spermatozoa corresponded to a subgroup of progressive spermatozoa exhibiting a higher amplitude of lateral head displacement. Although a high variability was detected among mice of different genotypes, the results of these experiments suggest that the percentage of spermatozoa in each category is similar in WT and *NPC2*$^{-/-}$ mice after 15 and 60 min capacitation (Fig. 5*b*).

It has been shown that the activation of second messengers that culminates in sperm protein phosphorylation is important for the acquisition of the fertilising capacity of spermatozoa. Thus, we investigated the ability of *NPC2*$^{-/-}$ spermatozoa to fertilise mouse eggs *in vitro*. Because gametes of the BALB/c strain, the original background for *NPC2*$^{-/-}$ mice, have a very low fertilisation efficiency *in vitro* (D. Busso, pers. comm.; Sztein *et al.* 2000), the *NPC2* mutation was transferred to a C57BL/6J background for seven generations before these studies were performed. Coincubation of capacitated spermatozoa from *NPC2*$^{-/-}$ mice with COCs resulted in significantly lower percentages of 2-cell embryos that those obtained after using spermatozoa from WT mice (Fig. 6*a*). Interestingly, when oocytes were denuded from the cumulus oophorus and the ZP, a modest yet significant increase in the number of eggs fertilised by *NPC2*$^{-/-}$ spermatozoa was observed (Fig. 6*b*). No parthenogenic 2-cell embryos were found after incubation of COCs or ZP-free eggs in the absence of spermatozoa.

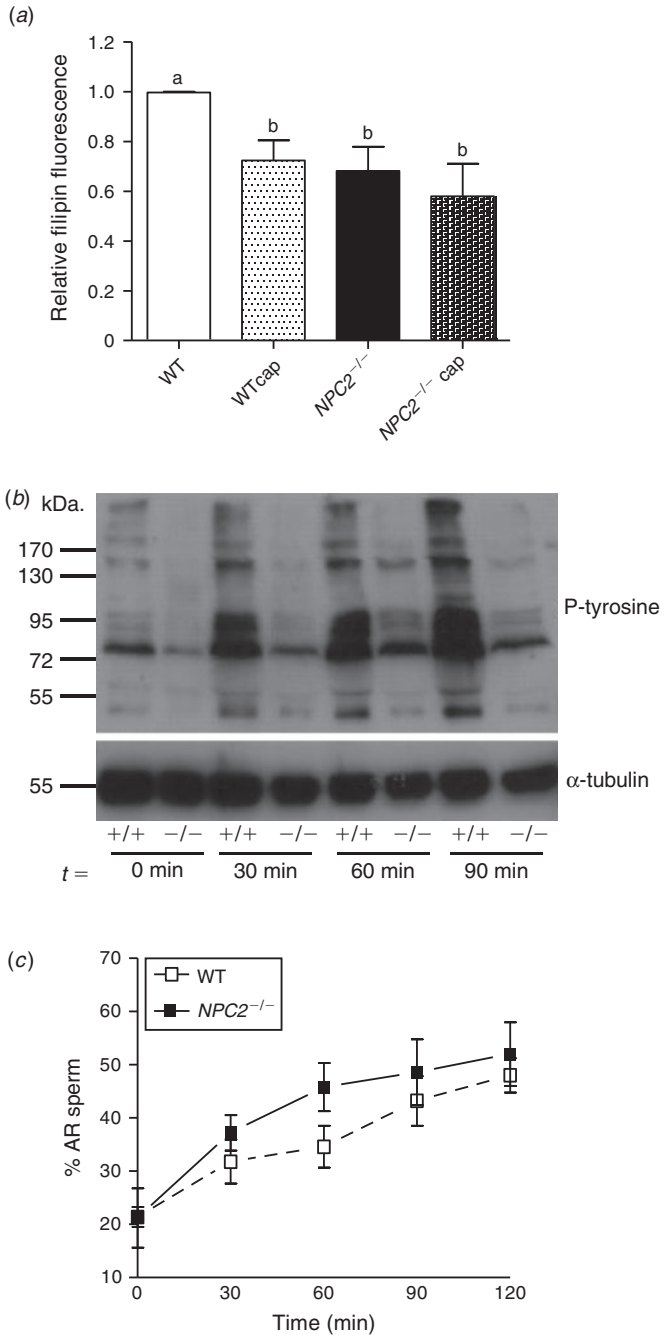
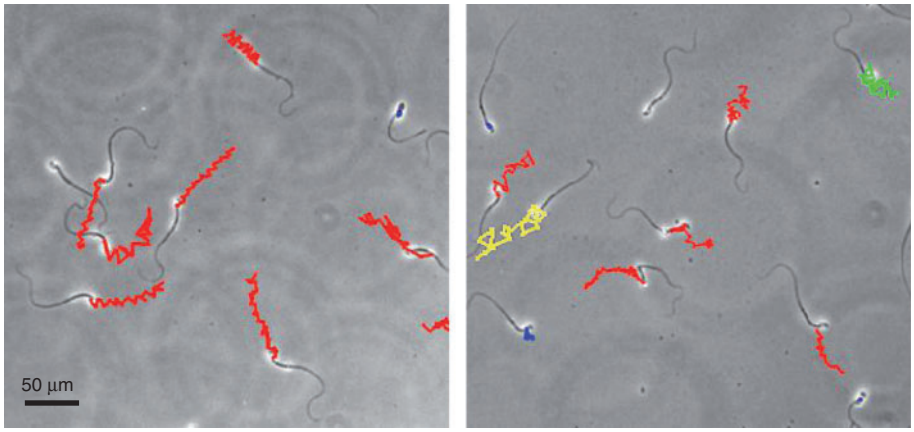


Fig. 4. Evaluation of cholesterol content and functional parameters in capacitated WT and *NPC2*$^{-/-}$ mice sperm. (*a*) Quantification of cholesterol in capacitated sperm from WT and *NPC2*$^{-/-}$ mice by flow cytometric analysis of filipin fluorescence ($n = 3$). (*b*) Western blot analysis of protein extracts from WT (+/+) and *NPC2*$^{-/-}$ (−/−) mice sperm incubated under capacitating conditions for different periods using anti-phosphotyrosine antibody and anti-α tubulin antibody as a loading control. A representative western blot is shown ($n = 3$). (*c*) Determination of the % of AR sperm by Coomasie Blue staining in WT and *NPC2*$^{-/-}$ capacitated cells. The results for 5 experiments using 100 sperm/experiment are shown as mean ± s.e.m. $^{*}P < 0.05$.

(*a*)



(*b*)

	Motility (%)			
Motility category	Capacitation 15 min		Capacitation 60 min	
	WT	*NPC2*$^{-/-}$	WT	*NPC2*$^{-/-}$
Progressive (red)	46 ± 16	37 ± 10	42 ± 12	28 ± 4
Slow (green)	20 ± 10	18 ± 6	14 ± 2	23 ± 4
Immotile (blue)	31 ± 6	43 ± 15	39 ± 12	45 ± 6
Hyperactivated (yellow)	3 ± 2	1 ± 1	5 ± 1	4 ± 1

Fig. 5. Analysis of motility in WT and *NPC2*$^{-/-}$ mice sperm. The motility of sperm incubated under capacitating conditions at 15 and 60 min was analysed by image processing algorithms. (*a*) Representative images at $t = 0$ including colour coded sperm path: progressive (red), slow (green), immotile (blue), and hyperactivated (yellow). (*b*) Percentages sperm motility in each category. The analysis corresponds to at least 3 sequences for each of the 3 mice analysed for each genotype.

Discussion

The abundance of NPC2 in the epididymis and its ability to transport cholesterol between membranes *in vitro* has led researchers to suggest an involvement of NPC2 in the regulation of cholesterol content in the sperm plasma membrane during epididymal maturation. The results of the present study show that NPC2-deficient mice have spermatozoa with less cholesterol and functional defects, providing, for the first time, direct evidence as to the relevance of NPC2 for mouse sperm function.

Our immunolocalisation studies showed that NPC2 in the mouse epididymis is present as various bands of approximately 20 kDa, as reported previously (Sleat *et al.* 2004; Klein *et al.* 2006). Although this band heterogeneity is probably due to different glycosylation forms of NPC2, further studies using glycosidases would be required to confirm this assumption, because other events, such as differential expression of the gene or proteolysis, could also account for the observations. As reported in other species, mouse NPC2 was detected in the three sections of the epididymis in a region-specific manner (Uhlenbruck *et al.* 1993; Fröhlich and Young 1996; Fouchécourt *et al.* 2000; Légaré *et al.* 2006). The presence of variable forms of NPC2 in the epididymis could be important for the localisation, function and/or stability of the protein, as has been shown previously for lysosomal NPC2 (Chikh *et al.* 2004). However, this point was beyond the scope of the present study.

Male *NPC2*$^{-/-}$ mice do not reproduce normally because they have locomotor problems when they reach sexual maturity and are unable to mate. These animals have normal reproductive organs and sperm morphology. However, epididymal spermatozoa from these mice have a reduced cholesterol content and exhibit defects in both signalling pathways related to capacitation and their ability to fertilise eggs *in vitro*.

The reduction in cholesterol content observed in spermatozoa from *NPC2*$^{-/-}$ mice was detected both biochemically and by filipin staining. Our flow cytometry experiments to quantify filipin fluorescence were performed after selecting the main population of PI-negative events. Preliminary analysis indicated that the proportion of non-sperm particles within this gated population was not negligible and accounted for approximately 25% of the particles detected. The analysis of filipin fluorescence in non-sperm and sperm subpopulations showed that filipin fluorescence by debris was low and the difference in filipin staining between WT and *NPC2*$^{-/-}$ spermatozoa was maintained after selecting for live cells. Although the results

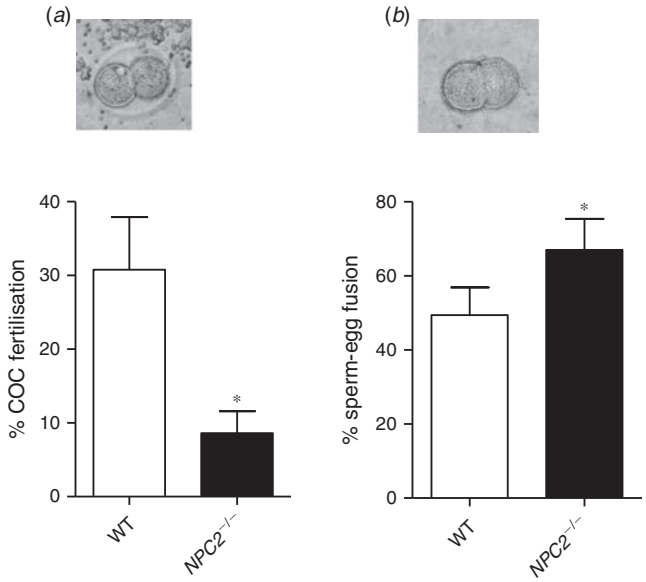


Fig. 6. Evaluation of WT and *NPC2*$^{-/-}$ mice sperm fertilising ability. Capacitated sperm were used to inseminate (*a*) cumulus-oocyte complexes and (*b*) denuded ZP-free oocytes, and the percentages of gamete fertilisation (in (*a*)) or fusion (in (*b*)) were determined by the presence of two-cell embryos the following day ($n \geq 4$; $^*P < 0.05$).

with or without consideration of debris were similar in this particular case, our future flow cytometry analyses will definitely include selection for live spermatozoa to avoid confounding results from non-sperm particles. Because live spermatozoa were used in these procedures and filipin is unable to permeate the plasma membrane in live spermatozoa, it is highly probable that the cholesterol defect in spermatozoa from *NPC2*$^{-/-}$ mice is at the level of the plasma membrane. Unlike what is observed in most cell types from patients with NPC disease or NPC-deficient animals, spermatozoa do not accumulate cholesterol because they do not possess functional lysosomes in their scarce cytoplasm. Thus, the deficiency of epididymal NPC2 is not expected to affect intracellular sperm cholesterol content, but to influence plasma membrane cholesterol given the absence of NPC2 in the epididymal fluid. With this in mind, the fact that spermatozoa obtained from the distal epididymis of *NPC2*$^{-/-}$ mice have less cholesterol than spermatozoa from WT mice suggests that NPC2 present in the epididymal fluid may be involved in the acquisition of cholesterol from the fluid by spermatozoa during maturation. However, it cannot be discounted that an accumulation of cholesterol in the lysosomes of testicular spermatogenic cells could also reduce the traffic of cholesterol to the plasma membrane and explain the low cholesterol content in spermatozoa. To evaluate this possibility it would be necessary to analyse the cholesterol content in spermatozoa obtained from purified testicular and caput populations from *NPC2*$^{-/-}$ mice. This is a subject for future studies in our laboratory. However, the fact that in WT mice NPC2 is so highly expressed in the epididymis supports its function as a cholesterol transfer molecule in this organ.

The low levels of cholesterol in spermatozoa from *NPC2*$^{-/-}$ mice was accompanied by different functional defects in those cells, supporting the idea of a close relationship between sperm lipid composition and function (Jones *et al.* 2007). Numerous studies indicate that the gradual removal of cholesterol during sperm transit along the female tract is one of the first key events initiating signalling cascades inducing capacitation. In this regard, the low cholesterol content and the reduced capacitation-induced cholesterol efflux in *NPC2*$^{-/-}$ spermatozoa could be related to defects in their ability to undergo tyrosine phosphorylation, an essential signal transduction pathway for fertilisation (Visconti *et al.* 1999*b*). Possibly, membrane fluidity and the distribution of molecules is altered in fresh, non-capacitated spermatozoa from *NPC2*$^{-/-}$ mice due to their constitutively lower cholesterol content, which affects the formation of capacitation-related signalling complexes during incubation under capacitation conditions. Despite the defect in tyrosine phosphorylation, spermatozoa obtained from *NPC2*$^{-/-}$ mice were capable of undergoing spontaneous AR as efficiently as spermatozoa from their WT littermates, and even in a slightly accelerated fashion, as indicated by the increase in the percentage of acrosome-reacted spermatozoa at 60 min. An increase in the lability and/or fluidity of the *NPC2*$^{-/-}$ sperm plasma membrane as a result of the lower cholesterol content could account for this observation. The normal levels of spontaneous AR despite the defective tyrosine phosphorylation detected in spermatozoa from *NPC2*$^{-/-}$ mice has also been observed in spermatozoa from other knockout mouse models for proteins involved in fertilisation, such as soluble adenylyl cyclase and epididymal cysteine rich protein 1 (Da Ros *et al.* 2004*b*; Xie *et al.* 2006), suggesting that the two events related to capacitation (i.e. tyrosine phosphorylation and AR) take place in a synchronised but independent manner. Although the percentage of hyperactivation may seem very low, it is within the range expected for spermatozoa from the C57BL/6J mice used in our experiments (Ghanayem *et al.* 2010; Goodson *et al.* 2011).

As expected, the functional defects found in the capacitation of spermatozoa from *NPC2*$^{-/-}$ mice also affect their ability to fertilise oocytes. Although the results obtained in the present study using ZP-intact and ZP-free eggs may seem contradictory, they are in perfect agreement with the 'cholesterol-deficient phenotype' observed in spermatozoa from *NPC2*$^{-/-}$ mice. Conversely, the reduction in the percentage fertilisation of ZP-intact eggs can be explained by defective tyrosine phosphorylation, because the activation of sperm-surface chaperones by tyrosine phosphorylation during capacitation has been shown to facilitate sperm–ZP binding (Asquith *et al.* 2004), which, in turn, is fundamental for the physiological induction of the AR and for the penetration of the ZP by spermatozoa. If biologically relevant, the modest enhancement of sperm–egg fusion in *NPC2*$^{-/-}$ mice could be in accordance with the presence of a more fluid, and possibly more fusogenic, plasma membrane exposed after the spontaneous AR.

Our results suggest that NPC2 is involved in the addition of cholesterol to spermatozoa during epididymal maturation. Curiously, previous studies that analysed the epididymal expression of human NPC2 and its behaviour in ejaculated spermatozoa from men undergoing vasectomy and vasovasostomy suggested

that this protein was involved in the removal of cholesterol from spermatozoa during epididymal transit (Legaré *et al.* 2004; Légaré *et al.* 2006). Although our results in mice and the findings described previously in the human and bovine support a role for NPC2 in cholesterol content remodelling during maturation, NPC2 function in mice appears to be opposite to that in the other two species. Although the reason for this difference is not clear, there are some experimental factors that could account for the differences. For example, analysis of sperm samples from ejaculates in bovine and human versus epididymal sperm samples in our case could bring about different interpretations as to the function of epididymal NPC2, because this protein has also been shown to be present in other mouse accessory glands, such as the prostate and seminal vesicles (results not shown). In addition, the incubation of bovine spermatozoa with purified NPC2 protein at a random concentration, which may differ from physiological concentrations, is also worth considering when discussing NPC2 function. In this regard, our studies comparing WT and NPC2-deficient mice were performed with highly homogeneous mouse sperm populations of epididymal origin, free from secretions from accessory gland fluids.

Previous evidence indicates the importance of NPC proteins for fertility in mice of both sexes. In females, a deficiency in NPC proteins was shown to induce defects in ovarian steroidogenesis that affect ovulation (Gévry *et al.* 2004; Busso *et al.* 2010). In addition, NPC1-knockout male mice were proven to be infertile due to partially arrested spermatogenesis, numerous sperm morphological defects and hypoandrogenaemia (Fan *et al.* 2006). Our studies show that *NPC2*$^{-/-}$ mice undergo normal spermatogenesis and produce normal spermatozoa. These results, together with preliminary testosterone determination assays indicating normal levels of testosterone (data not shown), suggest that testicular processes are not grossly affected in *NPC2*$^{-/-}$ mice.

The molecular mechanisms that explain the relevance of NPC2 for sperm function are still not clear. The impact of altered cholesterol metabolism in testicular germ cells on *NPC2*$^{-/-}$ sperm function cannot be ruled out due to the lack of evidence on caput sperm cholesterol levels. However, there are some lines of evidence supporting the idea that NPC2 present in the epydidimal fluid could be involved in the regulation of cholesterol levels in the sperm plasma membrane during maturation. First, NPC2 is one of the most abundant proteins found in the epididymal fluid, as well as in other lipid-rich physiological fluids, such as bile, plasma and milk, suggesting it may have a role in the extracellular space in addition to its well-known function within the lysosome. Interestingly, the lysosomal milieu and the epididymal fluid both have an acidic pH, and NPC2 activity is more efficient under this condition. Second, NPC2 expression in the epydidimis has been shown to be correlated with fertility in vasectomised patients (Legaré *et al.* 2006). Third, although purified NPC2 has been shown to be able to transfer cholesterol between membranes, it has also been shown to interact with the bovine sperm plasma membrane modulating its lipid composition (Infante *et al.* 2008; Girouard *et al.* 2008). Although the function for epididymal NPC2 is hypothetical at present, its elucidation could have important implications in the understanding of mammalian physiology and in the aid of human health.

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