Activated-farnesoid X receptor (FXR) expressed in human sperm alters its fertilising ability

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Abstract

The farnesoid X receptor alpha (FXR) is a bile acid sensor activated by binding to endogenous bile acids including chenodeoxycholic acid (CDCA). Although, FXR is expressed in male reproductive tissue, the relevance of the receptor on reproduction is scarcely known. Here, we demonstrated the FXR presence and its action on several human sperm features. Western blot and immunofluorescence assays evidenced the FXR expression in human spermatozoa and the localisation in the middle piece. CDCA increasing concentrations and GW4064, synthetic ligand of FXR, were used to study the FXR influence on sperm motility, survival, capacitation, acrosome reaction and on glucose as well as lipid metabolism. Interestingly, our data showed that increasing concentrations of CDCA negatively affected sperm parameters, while the receptor blockage by (Z)-Guggulsterone and by the anti-FXR Ab reversed the effects. Intriguingly, elevated CDCA levels increased triglyceride content, while lipase and G6PDH activities were reduced with respect to untreated samples, thus impeding the metabolic reprogramming typical of the capacitated sperm. In conclusion, in this study, we demonstrated for the first time a novel target for FXR and that the activated receptor alters the acquisition of sperm fertilising ability. We showed that sperm itself express the FXR and it is responsive to specific ligands of the receptor; therefore, bile acids influence this cell both in male and in female genital tracts. It might be hypothesized that bile acid levels could be involved in infertility with idiopathic origin as these compounds are not systematically measured in men undergoing medically assisted procreation.

Introduction

The farnesoid X receptor alpha (FXR or NR1H4) is a bile acid sensor, activated by binding to endogenous bile acids (BAs) (e.g., chenodeoxycholic acid (CDCA) and cholic acid (CA)). The FXR is member of the nuclear receptor superfamily of ligand-dependent transcription factors. Although FXR is typically referred as bile acid receptor, it is expressed in many tissues. High levels of FXR have been described not only in liver and intestine (Forman *et al.* 1995), but also in kidney and adrenals (Huber *et al.* 2002, Otte *et al.* 2003). In addition, low levels of FXR mRNA have also been reported in the heart, lung, stomach, adipose, thymus, spleen, ovary, testes and vascular tissue (Zhang *et al.* 2003, Bishop-Bailey *et al.* 2004, Higashiyama *et al.* 2008, Popescu *et al.* 2010, Vignozzi *et al.* 2011, Morelli *et al.* 2012). The FXR has a relevant participation, even directly or indirectly, in both male and female reproduction (Estela *et al.* 2015). According to initial reports, FXR seems not to be expressed in the testis (Forman *et al.* 1995, Seol *et al.* 1995). However, subsequent studies have suggested that FXR could be expressed in the testis of mice (Maeda *et al.* 2004) and certain reptiles (Alfaro *et al.* 2002).

Besides, FXR has been found in cells from male reproductive tissues such as Leydig cells, corpora cavernosa, epididymis, vas deferens, prostate, urethra and spermatogonia in humans and other mammals (Alfaro *et al.* 2002, Kaeding *et al.* 2008, Catalano *et al.* 2010, Vignozzi *et al.* 2011, Anaya-Hernández *et al.* 2014). Altogether, a role for FXR in the control of reproductive processes appears plausible; however, it remains to be demonstrated. Up to date, an action for FXR in the development of male sex organs and/or reproductive functions has been poorly investigated.

CDCA and CA are the major end products of bile acid biosynthesis (Vlahcevic *et al.* 1991, Chiang 1998) and Gray *et al.* have suggested that the bile acids are important for the testicular function by showing that FXR activation by CDCA affects the sex steroid production in Leydig cells (Gray & Squires 2013). Furthermore, the FXR activation by obeticholic acid, a semi-synthetic bile acid analogue, which has the chemical structure 6α-ethyl-chenodeoxycholic acid (INT-747) avoids the reduction of smooth musculature in the corpora cavernous and the erectile dysfunction promoted by a high-fat diet in animal models (Vignozzi *et al.* 2011). A similar effect has been observed in the bladder smooth musculature

of rats fed with a high-fat diet, in which the damage of the muscle induced by the diet is partially blunted by testosterone, but almost completely reverted by INT-747 (Morelli *et al.* 2012). Mice fed a diet supplemented with CDCA have a reduced fertility as consequence of testicular defects, a decreased protein accumulation of connexin-43 and N-cadherin and a high intra-testicular bile acid concentration (Baptissart *et al.* 2014). Few papers showed that BA has a direct action on sperm; however, it reduced sperm motility in a dose- and time-dependent way, in parallel with profound alterations of sperm ultrastructure (Psychoyos *et al.* 1993, Courtot *et al.* 1994). Classically, FXR forms heterodimers with 9-cis-retinoic acid receptor (RXRα) and binds to FXR-response elements (FXREs) (Modica *et al.* 2010); however, for several nuclear receptors a non-genomic action has been demonstrated also in sperm biology (Aquila *et al.* 2009*a*,*b*, Guido *et al.* 2011, Cappello *et al.* 2012, Aquila & De Amicis 2014, Rago *et al.* 2014). To date, no notice on a possible non-genomic actions for FXR have been reported; however, sperm acts through rapid effects given its peculiar structure and function. The increase of plasma BA levels is a common disturbance to several liver troubles from the earliest stages of the disease (Yamazaki *et al.* 2013). Data obtained from animal models suggest that the effects of liver disorders on male reproductive function might alter both endocrine and exocrine functions of the testis.

In this study, we have demonstrated for the first time that human sperm is a novel target for FXR, discovering an unsuspected field of action for this nuclear receptor. Interestingly, the FXR activated by high CDCA levels, negatively influenced different sperm features such as survival, motility, capacitation and acrosome reaction. Our finding provided new insight in the molecular mechanisms through which BAs exert their negative effects on human reproduction.

Materials and methods

Chemicals

EBSS medium, CDCA, (Z)-Guggulsterone, GW4064 and all other chemicals were purchased from Sigma Chemical. Acrylamide bisacrylamide was from Labtek Eurobio (Milan, Italy). Triton X-100 and eosin Y were from Farmitalia Carlo Erba (Milan, Italy). ECL Plus Western blotting detection system, Hybond TM ECL TM and Hepes sodium salt were from Amersham Pharmacia Biotech. Anti-FXR NB400 was from Novus Biologicals (NOVUS, Italy), anti FXR sc-25309, FXR blocking peptide, anti pAKT sc 514032, pMAPK sc 292838 and β-actin were purchased from Santa Cruz Biotechnology, peroxidase-coupled anti-rabbit IgG and anti-rabbit TRITC conjugate Ab were from Santa Cruz Biotechnology. Cholesterol oxidase (CHOD)–POD enzymatic colorimetric assay, triglycerides assay, lipase activity, glucose-6-phosphate dehydrogenase (G6PDH) activity, kits were purchased from Inter-Medical (Biogemina Italia Srl, Catania, Italy). Different controls were performed in the assay considered: Normal Rabbit Serum (NRS), BSA 2%, EtOH at 0.012% (used to dilute CDCA and GW4064 as well (Z)-Guggulsterone) did not give any effect in all the assays performed.

Semen samples and spermatozoa preparations

Human semen was collected, according to the World Health Organization (WHO)-recommended procedure (WHO 2010). Spermatozoa preparations were performed as previously described (De Amicis *et al.* 2011). Briefly, semen samples with normal parameters of volume, sperm count, motility, morphology and vitality according to the WHO Laboratory Manual (WHO 2010) were included in this study. The study has been approved by the local medical ethical committee, and all participants gave their informed consent.

Processing and treatments of human ejaculated sperm

For each experiment, the ejaculates of three different normozoospermic healthy donors were pooled and processed as previously described (De Amicis *et al.* 2011). Swim-up sperm purified, were washed with unsupplemented Earle's Balance Salt Solution medium (EBSS) without sodium bicarbonate, calcium and phenol red (uncapacitating medium) and re-suspended in the same medium. Then, it was incubated for 30 min at 37°C and 5% CO_2, without (control, NC) or with increasing CDCA (0.5, 2, 10, 50 and 100 μM). The minimum and maximum values of critical micellar concentration in water at 37°C for the sodium salts of CDCA are 3.0 mmol/L minimum and 30 mmol/L respectively (Bile acids: chemistry, physiology, and pathophysiology (Monte *et al.* 2009). Therefore, having used lower concentrations (μmol), the authors exclude a 'detergent/micellar' effect of CDCA.

It is important to point out that the concentrations 0.5, 2, 10, 50 and 100 μM are supra-physiological, however, when we treat with lower concentrations, no significant effects were observed. In another set of experiments, sperm were incubated with anti-FXR Ab, (Z)-Guggulsterone 10 μM (a specific inhibitor of FXR) and GW4064 6 μM (a specific activator of FXR). The concentration of GW4064 at 6 μM was utilized after carrying out a dose-response curve to estimate the reduction of sperm vitality after incubation with CDCA and GW4064 increasing concentrations (Supplementary Fig.1 (see section on Supplementary data at the end of the article)). When the anti-FXR Ab as well as the specific inhibitor (Z)-Guggulsterone were combined with CDCA, a pretreatment of 15 min was performed.

The anti-FXR Ab dilution of 1:100 was empirically determined to neutralise 97% of the FXR sperm sites into the incubation medium. Furthermore, additional controls were performed and the cells were treated with normal rabbit serum, BSA 2% and 0.015% of EtOH. CDCA was dissolved in ethanol (0.01% final concentration in the culture) and when used as solvent control did not induce positive results in all *in vitro* assays, obtaining data similar to that of the control.

Western blot analysis of sperm proteins

Each sperm sample was centrifuged for 5 min at 5000 **g**. The pellet was resuspended in lysis buffer (62.5 mmol/L Tris-HCl, pH 6.8; 150 mm NaCl; 2% SDS; 1% Triton X100; 10% glycerol; 1 mm phenylmethylsulfonylfluoride; 10 μg/mL leupeptin; 10 μg/mL aprotinin; 2 μg/mL pepstatin) as previously described (Zara *et al*. 2007, Madeo *et al*. 2009, Aquila *et al*. 2013). Briefly, an equal amount of proteins (70 μg) was boiled for 5 min, separated on an 11% polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes and probed with an appropriate dilution of the indicated primary Abs. The binding of the secondary Ab was revealed with the ECL Plus Western blotting detection system, according to the manufacturer's instructions. The negative control was performed using a sperm lysate that was immunodepleted of FXR (i.e. pre-incubated lysate with anti-FXR Abs for 1 h at room temperature and immunoprecipitated with Protein A/G-agarose). Furthermore, the specificity of anti-FXR antibodies was tested by pre-absorption of each primary antibody with an excess of the respective blocking peptide for 48 h at 4°C (negative controls). β-Actin served as a control for equal loading. Specifically, the intensity of the pAKT and pMAPK bands were normalised to the intensity of the actin band by densitometric analysis. pMAPK intensity was analysed only for the 42 kDa band.

Immunofluorescence labelling

Sperm cells were rinsed three times with 0.5 mM Tris–HCl buffer, pH 7.5 and allowed to settle onto slides in a humid chamber. The overlying solution was carefully pipetted off and replaced by absolute methanol for 7 min at −20°C. After methanol removal, sperm cells were washed in Tris-buffered saline (TBS) containing 0.1% Triton X-100 and were treated for immunofluorescence. Two anti-human FXR NB400 (1:200) and sc 25309 (1:100) were utilised as primary antibodies, and the anti-rabbit TRITC conjugated IgG (1:80) as secondary antibody. The specificity of anti-FXR antibodies was tested by pre-absorption controls (Rago *et al*. 2006, 2007).

The slides were examined under an Olympus BX41 microscope and the images were taken with CSV1.14 software, using a CAM XC-30 for image acquisition, observing a minimum of 200 spermatozoa for nine slides.

Sperm motility and viability

Sperm motility and viability were assessed by means of light microscopy examining an aliquot of each sperm sample, which had been incubated as mentioned earlier. Sperm motility was expressed as percentage of total motile spermatozoa, including the rapid progressive (PR) plus the slow progressive (NP) cells (normal values: PR + NP > 40% as reported by WHO 2010). Viability was assessed by red-eosin exclusion test using Eosin Y to evaluate potential toxic effects of the treatments. An independent observer scored 200 cells for stain uptake (dead cells) or exclusion (live cells). Sperm viability was expressed as the percentage of total live sperm. Viability was evaluated before and after pooling the samples, and there were no adverse effects among the different treatments on human sperm survival (Aquila *et al*. 2003, Cappello *et al*. 2012).

Measurement of cholesterol efflux

Cholesterol was measured in duplicate by a CHOD–POD enzymatic colorimetric method according to manufacturer's instructions in the incubation medium from human spermatozoa, as previously described (Aquila *et al*. 2006, 2009*a*, Santoro *et al*. 2013). Purified sperm samples, washed twice with uncapacitating medium, were incubated as mentioned earlier. At the end of the sperm incubation the culture media were recovered by centrifugation, lyophilised, and subsequently dissolved in 1 mL of buffer reaction. The samples were incubated for 10 min at room temperature, and then the cholesterol content was measured with the spectrophotometer at 505 nm. Cholesterol standard used was 200 mg/dL. The limit of sensitivity for the assay was 0.05 mg/dL. Inter- and intra-assay variations were 0.04% and 0.03%, respectively. Cholesterol results are presented as mg per 10×10^6 number of spermatozoa.

Acrosome reaction

Spermatozoa incubated with 0.5, 2, 10, 50 and 100 μM of CDCA, spermatozoa pre-incubated with anti-FXR Ab and then combined with 50 μM CDCA, spermatozoa incubated only with buffer (NC) were re-suspended in unsupplemented EBSS medium (5×10^6 sperm/mL), placed in a conical tube and cultured for 2 h in an atmosphere of 5% CO_2 in air at 37°C. Then, acrosomal status was monitored using the acrosome-specific fluorochrome fluorescein isothiocyanate-labelled peanut (*Arachis hypogaea*) agglutinin (FITC–PNA) in conjunction with DNA-specific fluorochrome propidium iodide (PI) as a viability test (Funahashi 2002). Briefly, sperm suspension (1×10^6 mL) was exposed to FITC–PNA (10 μg/mL) and PI (12 μmol/L) for 5 min at 37°C and then fixed by adding 1 μL of 12.5% (w/v) paraformaldehyde on 0.5 mol TRIS/L (pH 7.4). The slides were immediately examined with an epifluorescence microscope (Olympus BX41) with a multiple fluorescence filter (U-DM-DA/FI/TX2) observing a minimum of 200 spermatozoa per slide (100× objective). Acrosomal status was assessed according to the staining patterns.

Staining patterns

Spermatozoa with a nuclear red PI staining were considered as dead cells, while sperm cells without PI staining were considered as live cells. Live spermatozoa were classified into two main categories on the basis of the FITC–PNA staining as follows: (i) acrosome non-reacted cells with uniform green FITC–PNA fluorescence of acrosome cap; (ii) acrosome-reacted cells without any fluorescence and/or only equatorial segment staining. Values were expressed as percentage. Six replicate experiments were performed for each semen sample.

Assay of the G6PDH activity

The conversion of NADP+ to NADPH, catalysed by G6PDH, was measured by the increase in absorbance at 340 nm (Aquila *et al.* 2009a, 2013). Spermatozoa samples, washed twice with uncapacitating medium, were incubated in the same medium (control) for 30 min at 37°C and 5% CO_2. Other samples were incubated in the presence of the indicated treatments. After incubation, 50 µL of sperm extracts were loaded into individual cuvettes containing buffer (100 mM triethanolamine, 100 mM $MgCl_2$, 10 mg/mL glucose-6-phosphate, 10 mg/mL NADP+, pH 7.6) for spectrophotometric determination. The absorbance of samples was read at 340 nm every 20 s for 1.5 min. Data are expressed as nmol/min/10^6 spermatozoa. The enzymatic activity was determined with three control media: one without glucose-6-phosphate as substrate (G1), another without the coenzyme (NADP+) (G2) and the third without either substrate or coenzyme (G3).

Triglycerides assay

Triglycerides were measured in duplicate by a GPO–POD enzymatic colorimetric method according to manufacturer's instructions in sperm lysates and as previously described (Aquila *et al.* 2006, 2009a,b). Swim-up-purified sperm samples, washed twice by centrifugation with uncapacitating medium, were incubated in the same medium (control) for 30 min at 37°C and 5% CO_2. Other samples were incubated in the presence of the indicated treatments. At the end of sperm incubation, 10 µL of the lysate were added to 1 mL of the buffer reaction and incubated for 10 min at room temperature. Then, the triglycerides content was measured at 505 nm using a spectrophotometer. Data are presented as mg/10^6 sperms.

Lipase activity assay

Lipase activity was evaluated, by the method of Panteghini (Panteghini *et al.* 2001) based on the use of 1,2-o-dilauryl-rac-glycero-3-glutaric acid-(6′-methylresorufin) ester (DGGR) as substrate, as previously described (Aquila *et al.* 2006, 2009b, De Amicis 2012). Fifty micrograms of sperm extracts were loaded into individual cuvettes containing buffer for spectrophotometric determination. DGGR is cleaved by lipase, resulting in an unstable dicarbonic acid ester, which is spontaneously hydrolysed to yield glutaric acid and methylresorufin, a bluish-purple chromophore with peak absorption at 580 nm. The absorbance of samples was read every 20 s for 1.5 min. The rate of methylresorufin formation is directly proportional to the lipase activity in the sample. Analysis of total imprecision gave a coefficient of variation between 0.02% and 0.032%. The estimated reference interval was 6–38 U/L (µmol/min/mg protein). The enzymatic activity was determined with three control media: one without the substrate, another without the co-enzyme (colipase) and the third without either substrate or co-enzyme.

Statistical analysis

The Western blotting and immunofluorescence analyses were performed in at least four independent experiments.

The data obtained from motility, viability and acrosome reaction assays (six replicate experiments using duplicate determinations) were presented as the mean ± S.E.M. The data obtained from cholesterol efflux, triglycerides assay, lipase activity and G6PDH activity (six replicate experiments using duplicate determinations), were presented as the mean ± S.E.M. Each data point of the Western blotting densitometric analysis representing the band intensities and evaluated in terms of arbitrary densitometric units were presented as the mean ± S.E.M. The differences in mean values were calculated using analysis of variance (ANOVA) with a significance level of $P < 0.05$. The Mann–Whitney *U* tests was used after ANOVA as *post hoc* test.

Results

Human sperm express FXR

FXR expression was first investigated by Western blotting analysis, using two different antibodies and obtaining the same result. A single immune-reactive band at about 60 kDa in two different sperm samples lysates from human sperm (N1, N2) was observed (Fig. 1A). MCF7 (breast cancer cell line) was used as positive control (Catalano *et al.* 2010), while no band was observed in the negative controls performed as reported in 'Materials and methods' section (Fig. 1A and A1).

FXR is located in the midpiece

Given the extreme polarisation of sperm cell, it is important to investigate the location of a molecule since it may be indicative of the function. A brilliant red light revealed the FXR presence only in the
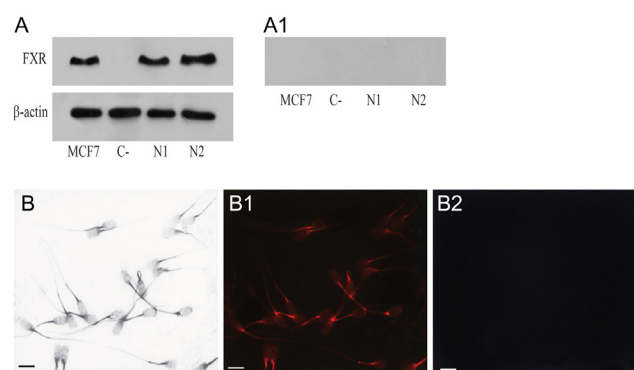

Figure 1 FXR expression in human spermatozoa. (A) Representative experiment of pooled sperm samples (lanes N1, N2); MCF7 breast cancer cells as positive control (MCF7), (C−) negative control; (A1) negative pre-absorption control. Actin serves as a loading control. The experiments were repeated at least four times and images show the results of one representative experiment. FXR immunolabelling of human sperm. (B) Phase contrast; (B1) expression of FXR in human spermatozoa showed a red immunofluorescence labelling in the sperm mid piece; (B2) negative pre-absorption control. Pictures are representative of four similar experiments. Scale bars: 5 µM.

sperm midpiece (Fig. 1B1). No fluorescent signal was obtained when primary antibody was omitted (negative pre-absorption control), thus confirming the specificity of the antibody binding. By using two different primary antibodies the same pattern of localisation was observed.

CDCA through FXR negatively influences sperm motility and survival

Assessment of the motile sperm fraction is perhaps the most widely used measure of semen quality, since motility describes the ability of sperm to move properly towards an egg. Therefore, we investigated the effect of increasing CDCA from 0.5 to 100 µM. As shown in Fig. 2A, sperm motility was significantly reduced after CDCA treatment, whereas the combined treatment with Ab anti-FXR plus 50 µM CDCA or (Z)-Guggulsterone plus 50 µM CDCA restored the motility.

Another important parameter of human sperm performance consists in the capacity of sperm to survive as long as possible to have the chance to find and fertilise the oocyte. From our data, CDCA treatment inhibits sperm survival, while the pretreatment with Ab anti-FXR or (Z)-Guggulsterone inhibited the CDCA-induced effects (Fig. 2B). It appears that CDCA was more effective than GW4064, the specific synthetic FXR activator, in inhibiting motility and survival.

FXR affects AKT and MAPK phosphorylation

To further define the FXR-induced effect on sperm survival, we analysed this action from a molecular point of view, exploring the main signalling involved in cell survival and previously explored in human sperm, the PI3K/Akt pathway (Aquila *et al.* 2007) and MAPK 42/44 phosphorylations (Santoro *et al.* 2013). Our data showed that pAKT and prevalently the p42 band of pMAPK were significantly reduced by increasing CDCA and GW4064 6 µM treatments (Fig. 3). This effect was reversed by (Z)-Guggulsterone.

CDCA did not induce cholesterol efflux in human sperm

Sperm functional maturation *in vivo* occurs during its travel in the female genital tract, where sperm undergo the capacitation process. A main feature of the capacitation comprises the cholesterol efflux by sperm, and the role of FXR in regulating cholesterol homeostasis is well established. Therefore, we investigated a possible action of FXR in the capacitation, by treating sperm with increasing CDCA or GW4064 6 µM concentrations. As shown in the Fig. 4A, CDCA reduced cholesterol efflux, although in a significant way at 50 and 100 µM. (Z)-Guggulsterone reversed the effect.
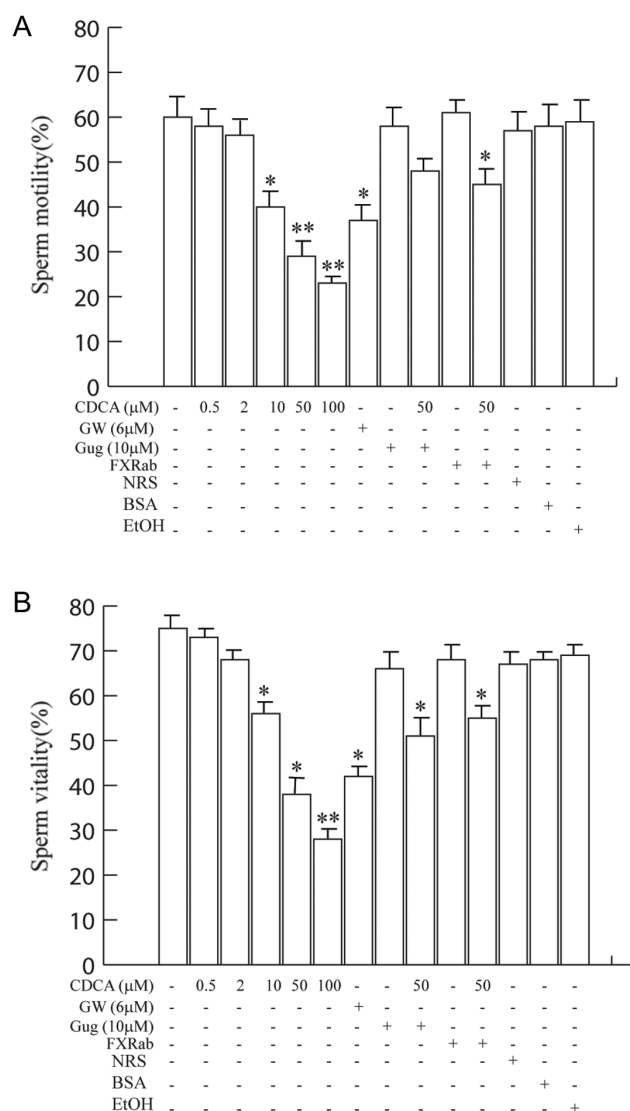

Figure 2 FXR influences on sperm motility and survival. (A) Sperm motility, (B) sperm vitality after CDCA (0.5, 2, 10, 50 and 100 µM), GW (6 µM), Gug (10 µM) with or without 50 µM CDCA, + FXR Ab with or without 50 µM CDCA, NRS, BSA and EtOH. NC, non-capacitated sperm. Columns represent mean ± s.e.m. of six independent experiments each done in duplicate, *$P < 0.05$ vs control, **$P < 0.001$ vs control.

CDCA through FXR produced acrosome reaction failure

The full acquisition of sperm ability to fertilise culminates with the acrosome reaction, which is the end point of fully capacitated sperm. However, premature acrosome loss and/or acrosome reaction failure are also important causes of male infertility. Figure 4B shows a representative fluorescence pattern of human spermatozoa, stained with FITC–PNA + PI for the assessment of acrosome status and viability as previously reported (Carpino *et al.* 2010). The CDCA treatment, increased the incidence of dead spermatozoa (PI positive cells) as well as the acrosome non-reacted cells, of consequence the
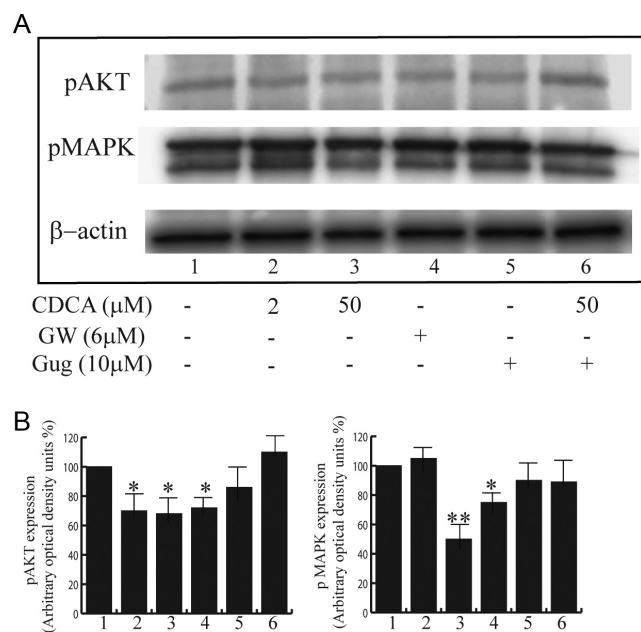
Figure 3 *pAKT e pMAPK* activation in human spermatozoa. Western analyses of pAKT, and pMAPK were performed on 70 µg of total protein. (A) Representative experiment of pooled sperm samples (*lane 1* untreated sperm cells); (*lanes 2, 3* sperm cells incubated with CDCA 2 and 50 µM respectively); (*lane 4* sperm incubated with GW 6 µM); (*lane 5 and 6* sperm cell treated with Gug 10 µM without/with CDCA 50 µM). (B) Columns are mean of four independent experiments in which band intensities were evaluated in terms of optical density arbitrary units. Actin serves as a loading control.

effects of CDCA on live sperm acrosome reaction are drastically reduced. By pre-treating with anti-FXR Ab or (Z)-Guggulsterone, the results were similar to that of the control (Fig. 4B1). It appears that the CDCA treatment through FXR impedes acrosome reaction.

Effects of CDCA/FXR on lipid metabolism in human sperm

During sperm extra-testicular maturation, an overall increase in sperm metabolism occurs and in somatic cells, FXR is an important regulator of lipid and glucose metabolism (Catalano *et al*. 2010). As shown in Fig. 5A, we first investigated triglycerides intracellular content upon the treatments indicated in the figure. Our data evidenced a significant increase in the triglycerides content, whereas the pre-treatment with anti-FXR Ab or (Z)-Guggulsterone plus 50 µM CDCA attenuated the effect. GW4064 6 µM also increased the triglycerides levels although in a lesser extent with respect CDCA 50 µM. In the same experimental conditions, the lipase activity decreased (Fig. 5B), indicating a reduction in the lipid utilisation, according to the increased levels of the triglycerides. In this context the pre-treatment with anti-FXR Ab significantly reversed the CDCA effect, while
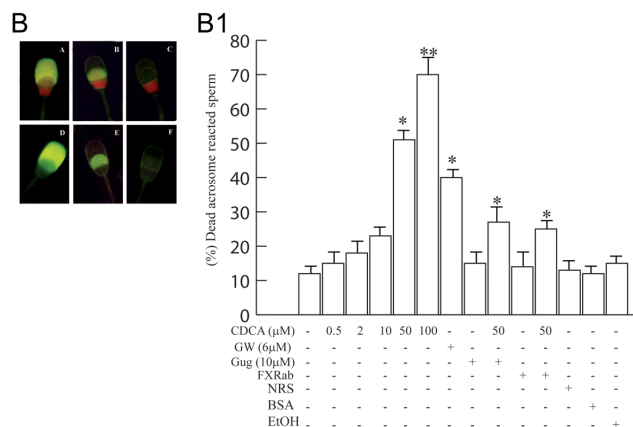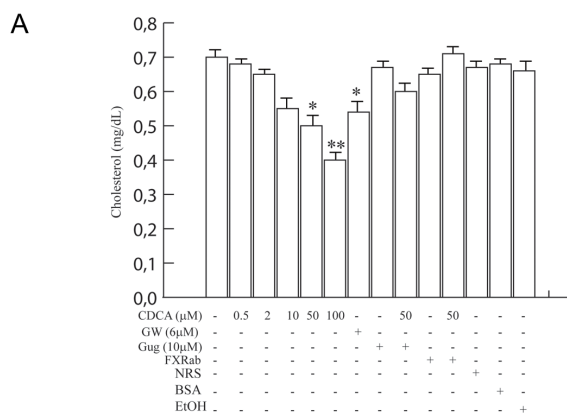

Figure 4 FXR affects sperm capacitation and acrosome reaction. (A) Sperm cholesterol efflux after the previous indicated (Fig. 2) treatments. NC, non-capacitated sperm. Columns represent mean ± s.e.m. of six independent experiments each done in duplicate, *$P < 0.05$ vs control, **$P < 0.001$ vs control. (B) Acrosome reaction staining pattern with FITC–PNA + P: A and B dead non-reacted spermatozoa, C dead reacted sperm, D and E live non-reacted spermatozoa, F live reacted sperm. (B1) Dead reacted sperm after CDCA treatment. Columns represent mean percentage ± s.e.m. of six independent experiments. *$P < 0.05$ vs control, **$P < 0.001$ vs control.

(Z)-Guggulsterone plus 50 µM CDCA slightly inhibited the CDCA action.

CDCA reduces glucose metabolism through the pentose phosphate pathway

To further investigate the role of FXR in sperm metabolism, we studied glucose metabolism through the pentose phosphate pathway (PPP). The evaluation of the G6PDH activity, which represents a key enzyme of this way, has been shown to be crucial in the acquisition of fertilising capability as well as to mediate gamete fusion (Aquila *et al*. 2005). From our results, it emerges that CDCA and GW4064 6 µM significantly reduced the G6PDH activity (Fig. 6). Interestingly, the pre-treatment with anti-FXR Ab or (Z)-Guggulsterone plus 50 µM CDCA produced a higher effect than the control in sperm treated with CDCA in the presence of the anti-FXR.
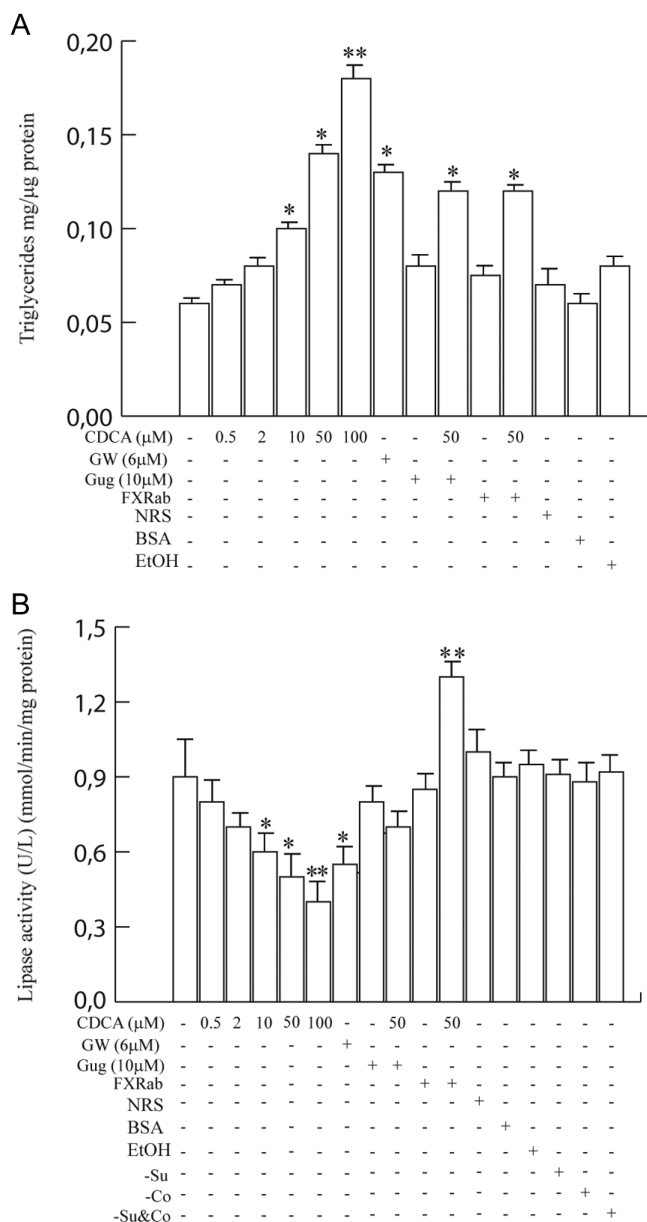
A

Triglycerides mg/µg protein

CDCA (µM)	-	0.5	2	10	50	100	-	-	50	-	50	-	-	-	-
GW (6µM)	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
Gug (10µM)	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-
FXRab	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-
NRS	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
BSA	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
EtOH	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+

B

Lipase activity (U/L) (mmol/min/mg protein)

CDCA (µM)	-	0.5	2	10	50	100	-	-	50	-	50	-	-	-	-	-	-
GW (6µM)	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
Gug (10µM)	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-
FXRab	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-
NRS	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
BSA	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
EtOH	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
-Su	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
-Co	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
-Su&Co	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+

Figure 5 FXR alters sperm lipid metabolism. (A) Sperm tryglicerides levels; (B) lipase activity after the previous indicated treatments (Fig. 2), furthermore, the lipase activity was determined with three control media: one without the substrate (-Su), another without the colipase (-Co) and the third without either substrate or co-enzyme (-Su and Co). NC, non-capacitated sperm. Columns represent mean ± s.e.m. of six independent experiments each done in duplicate. $*P<0.05$, $**P<0.001$ vs control.

G6PDH activity

CDCA (µM)	-	0.5	2	10	50	100	-	-	50	-	50	-	-	-	-	-	-
GW (6µM)	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
Gug (10µM)	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-
FXRab	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-
NRS	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
BSA	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
EtOH	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
G1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
G2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
G3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+

Figure 6 FXR reduces sperm glucose metabolism through the PPP. (A) Sperm G6PDH activity after the previous indicated treatments (Fig. 2). NC, non-capacitated sperm. The enzymatic activity was determined with three control media: one without glucose-6-phosphate as substrate (G1), another without the coenzyme (NADP+) (G2) and the third without either substrate or coenzyme (G3). Columns represent mean ± s.e.m. of six independent experiments each done in duplicate, $*P<0.05$ vs control, $**P<0.001$ vs control.

Discussion

Infertility affects approximately 10/15% of all couples worldwide and male factors contribute nearly 30–55% of all cases of infertility (Callister 2010). As it concerns male fertility disorders, the aetiology remains elusive and 25% of these cases are idiopathic. A growing number of studies sust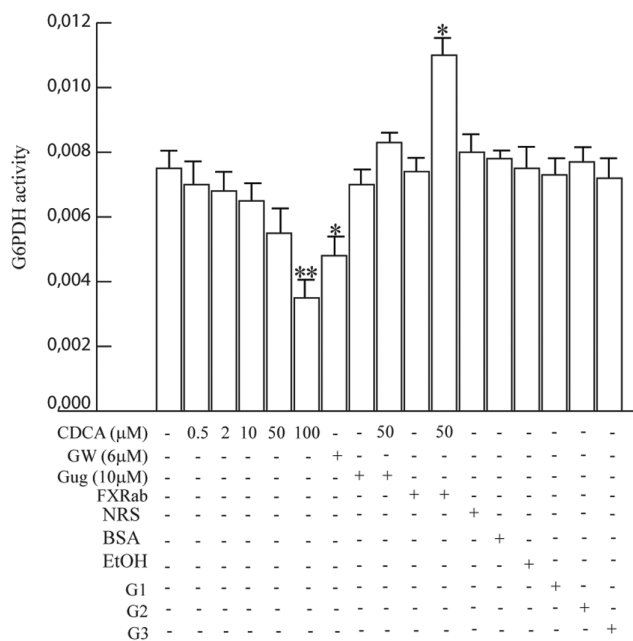ain the idea that alteration of BAs homeostasis could impact testicular physiology and male fertility. BAs have been described as molecules that signal through the nuclear FXR (Makishima *et al.* 1999, Parks *et al.* 1999, Wang *et al.* 1999), thus the FXR action in the regulation of male fertility has been hypothesised (Sèdes *et al.* 2017). Herein, we showed for the first time the FXR expression in human sperm and its localisation in the midpiece. Besides, FXR activation by specific ligands such as CDCA or a synthetic agonist, GW4064, deregulated several sperm functional features.

In our study, we showed that FXR is expressed in human sperm by Western blotting and immunofluorescence analyses. Expression of FXR in the male gamete is a novel intriguing finding since it may have an important role in the regulation of sperm activities. The immunocytochemical data corroborating the Western blot analysis evidenced the localisation of FXR specifically in the middle piece where sperm contains the mitochondria. Taking into account the extreme polarisation of sperm cell, the position of a molecule may be indicative of its role; therefore, FXR in our context might have an action on sperm metabolism.

FXR is activated by BAs and several studies identified particularly CDCA, as the most potent endogenous ligand for FXR (Zhang 2010). By evaluating the effects of increasing CDCA and of GW4064 6 µM, a specific synthetic activator of FXR, on human sperm motility

and viability, we showed a negative effect on these peculiar sperm functions. It is important to point out that the concentrations we used are supra-physiological, and this may account for a cytotoxic effect of elevated concentration of CDCA on human spermatozoa. Indeed, the potential link between BAs concentrations and male fertility disorders has been reported in several liver troubles where the increase of BA plasma levels is a common disturbance from the earliest stages of the disease (Yamazaki *et al.* 2013). In fact, during the last decade, several studies have reported the links between BA signalling pathways, male testicular physiology and subsequent fertility disorders. Given the importance of cholesterol regulation into sperm capacitation and the implication of FXR in this process in other tissues, we investigated whether the receptor is implicated in this sperm feature. Intriguingly, from our data, it emerges that both the FXR-agonists used in the study, CDCA and GW4064, were able to break capacitation and acrosome reaction processes, really exclusive of the sperm cell. All these effects were reversed, at least in part, by using anti-FXR Ab as well as the FXR-specific synthetic inhibitor (Z)-Guggulsterone, discovering a novel and unsuspected field of action for this nuclear receptor.

BAs/FXR signalling is able to modulate lipid, glucose and energy homeostasis (Mangelsdorf *et al.* 1995, Claudel *et al.* 2005, Kalaany & Mangelsdorf 2006, Scotti *et al.* 2007). Bile salts exert a broad regulatory role in systemic lipid metabolism via FXR, and gallstone patients treated with CDCA showed decreased plasma triglyceride levels (Ghosh Laskar *et al.* 2017). Conversely in our study, we observed an increase of the triglyceride levels, in agreement with a reduced lipase activity. The FXR blockage by pre-treating with the specific Ab anti-FXR or (Z)-Guggulsterone did not affect the triglyceride content as well as the lipase activity. Instead, it seems that activated FXR exerted a lipogenetic effect on human sperm lipid metabolism.

BAs also modulate glucose homeostasis, reducing blood glucose levels and activating the PPP (Tiangang *et al.* 2012). In human sperm, the beneficial effect of glucose on the acquisition of fertilising ability on gamete fusion is mediated by glucose metabolism through the PPP (Urner & Sakkas 1999*a*, Urner *et al.* 2001), where G6PDH is the key rate-limiting enzyme that regulates the production of NADPH (Urner & Sakkas 1999*b*). From our data, the G6PDH activity was significantly reduced, while the immune-neutralisation of the receptor as well as the use of (Z)-Guggulsterone abated the effect, indicating that the glucose metabolism through the PPP is slowed. Sperm, during his lifetime, passes through two different physiological stages: a steady state, as uncapacitated, during which the gamete economises and/or stores energy, and a state of functional maturation, during which the gamete becomes precisely capacitated with considerable energy expenditure. Generally, it might be believed that the uncapacitated gamete is

associable to an anabolic metabolism, while in the capacitated state to a catabolic one. Capacitated sperm display an increased metabolic rate and overall energy expenditure, presumably to affect the changes in sperm signalling and function during the capacitation process. From our data, it appears that activated-FXR impedes the switch of sperm into the capacitation, since it decreases both glucose and lipid metabolism.

Furthermore, in our finding, CDCA seems to be more effective with respect GW4064 in sperm and this may be consistent with the higher affinity of CDCA for FXR, which has been observed in MCF7, MDA-MB 231 and MDA-MB468 breast cancer cell lines (Alasmael *et al.* 2015). This could be also due to the concentration of the agonist we used.

Interestingly, we can establish the physiopathologic correlates of our findings considering that the association between liver disorders and fertility troubles has been validated and specified using several experimental models. Bile duct ligation in rat or chicken males leads to bile accumulation in the liver (Kiani *et al.* 2009), related with a loss of hepatocytes function and steatosis development, characteristics of cholestatic disorders (Houten 2007). These findings have also highlighted a decrease in plasma testosterone concentration independent of the hypothalamo–pituitary axis and associated with a loss of the germ cells. Therefore, data obtained from animal models suggest that the effects of liver disorders on male reproductive function might alter both endocrine and exocrine functions of the testis. While it is well known that liver disorders are pathological conditions in which BA concentration reached high levels, not much is known about the molecular links between liver diseases and male fertility disorders. In this study, we think that an important link between liver disorders and male infertility has been highlighted.

Furthermore, derivatives of BAs have been proposed as molecules for the treatment of diseases such as diabetes or obesity. However, the consequences of a long-term exposure to BAs signalling molecules in mouse models, although improved some metabolic syndrome parameters, impaired male fertility and testicular physiology (Watanabe *et al.* 2006, Vega *et al.* 2015). In addition, BAs can also have deleterious effects on organs involved in post-testicular maturation such as the epididymis or seminal vesicles. Indeed, it has been shown that a BA enriched diet induces a decrease of the seminal vesicles weight (Baptissart *et al.* 2014).

Collectively, these observations and our data support and highlight the proposition that BAs/FXR signalling pathways play a role on fertility troubles and could participate to infertility. Particularly, our study evidenced that sperm itself express the FXR, and it is responsive to specific ligands of the receptor, therefore, BAs impact this cell both in male and in female genital tracts. It might be hypothesised that BA levels could be involved in infertility with idiopathic origin as these compounds

are not systematically measured in men undergoing to assisted medically assisted procreation.

Supplementary data

This is linked to the online version of the paper at https://doi.org/10.1530/REP-18-0203.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References

Alasmael N, Mohan R, Meira LB, Swales KE & Plant NJ 2015 Activation of the Farnesoid X-receptor in breast cancer cell lines results in cytotoxicity but not increased migration potential *Cancer Letters* **370** 250–259. (https://doi.org/10.1016/j.canlet.2015.10.031)

Alfaro JM, Ricote M, Lobo MV, Royuela M, Fraile B, Paniagua R & Arenas MI 2002 Immunohistochemical detection of the retinoid acid receptors (RXR-alpha, -beta, -gamma) and Farnesoid X-activated receptor (FXR) in the marbled newt along the annual cycle. *Molecular Reproduction and Development* **62** 216–222. (https://doi.org/10.1002/mrd.10104)

Anaya-Hernández A, Méndez-Tepepa M, Hernández-Aragón LG, Pacheco P, Martínez-Gómez M, Castelán F & Cuevas E 2014 Farnesoid X receptor immunolocalization in reproductive tissues of adult female rabbits. *Acta Histochemica* **116** 1068–1074. (https://doi.org/10.1016/j.acthis.2014.05.003)

Aquila S & De Amicis F 2014 Steroid receptors and their ligands: effects on male gamete functions. *Experimental Cell Research* **328** 303–313. (https://doi.org/10.1016/j.yexcr.2014.07.015)

Aquila S, Sisci D, Gentile M, Carpino A, Middea E, Catalano S, Rago V & Andò S 2003 Towards a physiological role for cytochrome P450 aromatase in ejaculated human sperm. *Human Reproduction* **18** 1650–1659. (https://doi.org/10.1093/humrep/deg340)

Aquila S, Gentile M, Middea E, Catalano S, Morelli C, Pezzi V & Andò S 2005 Leptin secretion by human ejaculated spermatozoa. *Journal of Clinical Endocrinology and Metabolism* **90** 4753–4761. (https://doi.org/10.1210/jc.2004-2233)

Aquila S, Bonofiglio D, Gentile M, Middea E, Gabriele S, Belmonte M, Catalano S, Pellegrino M & Ando S 2006 Peroxisome proliferator-activated receptor (PPAR)gamma is expressed by human spermatozoa: its potential role on the sperm physiology. *Journal of Cellular Physiology* **209** 977–986. (https://doi.org/10.1002/jcp.20807)

Aquila S, Middea E, Catalano S, Marsico S, Lanzino M, Casaburi I, Barone I, Bruno R, Zupo S & Andò S 2007 Human sperm express a functional androgen receptor: effects on PI3K/AKT pathway. *Human Reproduction* **22** 2594–2605. (https://doi.org/10.1093/humrep/dem243)

Aquila S, Guido C, Middea E, Perrotta I, Bruno R, Pellegrino M & Andò S 2009*a* Human male gamete endocrinology: 1alpha, 25-dihydroxyvitamin D3 (1,25(OH)2D3) regulates different aspects of human sperm biology and metabolism. *Reproductive Biology and Endocrinology* **7** 140. (https://doi.org/10.1186/1477-7827-7-140)

Aquila S, Guido C, Laezza C, Santoro A, Pezzi V, Panza S, Andò S & Bifulco M 2009*b* A new role of anandamide in human sperm: focus on metabolism. *Journal of Cellular Physiology* **221** 147–153. (https://doi.org/10.1002/jcp.21837)

Aquila S, Santoro M, De Amicis F, Guido C, Bonofiglio D, Lanzino M, Cesario MG, Perrotta I, Sisci D & Morelli C 2013 Red wine consumption may affect sperm biology: the effects of different concentrations of the phytoestrogen Myricetin on human male gamete functions. *Molecular Reproduction and Development* **80** 155–165. (https://doi.org/10.1002/mrd.22145)

Baptissart M, Vega A, Martinot E, Pommier AJ, Houten SM, Marceau G, de Haze A, Baron S, Schoonjans K, Lobaccaro JM *et al.* 2014 Bile acids alter male fertility through G-protein-coupled bile acid receptor 1 signaling pathways in mice. *Hepatology* **60** 1054–1065. (https://doi.org/10.1002/hep.27204)

Bishop-Bailey D, Walsh DT & Warner TD 2004 Expression and activation of the farnesoid X receptor in the vasculature. *PNAS* **101** 3668–3673. (https://doi.org/10.1073/pnas.0400046101)

Callister LC 2010 Global infertility: are we caring yet? *MCN: The American Journal of Maternal/Child Nursing* **35** 174. (https://doi.org/ 10.1097/NMC.0b013e3181d760be)

Cappello AR, Guido C, Santoro A, Santoro M, Capobianco L, Montanaro D, Madeo M, Andò S, Dolce V & Aquila S 2012 The mitochondrial citrate carrier (CIC) is present and regulates insulin secretion by human male gamete. *Endocrinology* **153** 1743–1754. (https://doi.org/10.1210/en.2011-1562)

Carpino A, Rago V, Guido C, Casaburi I & Aquila S 2010 Insulin and IR-beta in pig spermatozoa: a role of the hormone in the acquisition of fertilizing ability. *International Journal of Andrology* **33** 554–562. (https://doi.org/10.1111/j.1365-2605.2009.00971.x)

Catalano S, Malivindi R, Giordano C, Gu G, Panza S, Bonofiglio D, Lanzino M, Sisci D, Panno ML & Andò S 2010 Farnesoid X receptor, through the binding with steroidogenic factor 1-responsive element, inhibits aromatase expression in tumor Leydig cells. *Journal of Biological Chemistry* **285** 5581–5593. (https://doi.org/10.1074/jbc.M109.052670)

Chiang JYL 1998 Regulation of bile acid synthesis. *Frontiers in Bioscience* **3** D176–D193. (https://doi.org/10.2741/A273)

Claudel T, Staels B & Kuipers F 2005 The farnesoid X receptor – a molecular link between bile acid and lipid and glucose metabolism. *Arteriosclerosis, Thrombosis, and Vascular Biology* **25** 2020–2030. (https://doi.org/10.1161/01.ATV.0000178994.21828.a7)

Courtot AM, Nikas G, Gravanis A & Psychoyos A 1994 Effects of cholic acid and 'Protectaid' formulations on human sperm motility and ultrastructure. *Human Reproduction* **9** 1999–2005. (https://doi.org/10.1093/oxfordjournals.humrep.a138382)

De Amicis F, Guido C, Perrotta I, Avena P, Panza S, Andò S & Aquila S 2011 Conventional progesterone receptors (PR) B and PRA are expressed in human spermatozoa and may be involved in the pathophysiology of varicocoele: a role for progesterone in metabolism. *International Journal of Andrology* **34** 430–445. (https://doi.org/10.1111/j.1365-2605.2010.01111.x)

De Amicis F, Santoro M, Guido C, Sisci D, Bruno R, Carpino A & Aquila S 2012 Progesterone through progesterone receptors affects survival and metabolism of pig sperm. *Animal Reproduction Science* **4320** 287–284. (https://doi.org/ 10.1016/j.anireprosci.2012.09.004)

Estela C, Martínez-Gómez M & Castelán F 2015 Farnesoid X receptor and reproduction. *Receptors and Clinical Investigation* **2** e463. (https://doi.org/10.14800/rci.463)

Forman BM, Goode E, Chen J, Oro AE, Bradley DJ, Perlmann T, Noonan DJ, Burka LT, McMorris T, Lamph WW *et al.* 1995 Identification of a nuclear receptor that is activated by farnesol metabolites. *Cell* **81** 687–693. (https://doi.org/10.1016/0092-8674(95)90530-8)

Funahashi H 2002 Induction of capacitation and the acrosome reaction of boar spermatozoa by L-arginine and nitric oxide synthesis associated with the anion transport system. *Reproduction* **124** 857–864. (https://doi.org/10.1530/rep.0.1240857)

Ghosh Laskar M, Eriksson M, Rudling M & Angelin B 2017 Treatment with the natural FXR agonist chenodeoxycholic acid reduces clearance of plasma LDL whilst decreasing circulating PCSK9, lipoprotein(a) and apolipoprotein C-III. *Journal of Internal Medicine* **281** 575–585. (https://doi.org/10.1111/joim.12594)

Gray MA & Squires EJ 2013 Effects of nuclear receptor transactivation on steroid hormone synthesis and gene expression in porcine Leydig cells. *Journal of Steroid Biochemistry and Molecular Biology* **133** 93–100. (https://doi.org/ 10.1016/j.jsbmb.2012.09.014)

Guido C, Perrotta I, Panza S, Middea E, Avena P, Santoro M, Marsico S, Imbrogno P, Andò S & Aquila S 2011 Human sperm physiology: estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) influence sperm metabolism and may be involved in the pathophysiology of varicocele-associated male infertility. *Journal of Cellular Physiology* **226** 3403–3412. (https://doi.org/10.1002/jcp.22703)

Higashiyama H, Kinoshita M & Asano S 2008 Immunolocalization of farnesoid X receptor (FXR) in mouse tissues using tissue microarray. *Acta Histochemica* **110** 86–93. (https://doi.org/10.1016/j.acthis.2007.08.001)

Houten SM 2007 A liver revival featuring bile acids. *Journal of Hepatology* **46** 539–540 (https://doi.org/10.1016/j.jhep.2006.12.005)

Huber RM, Murphy K, Mian B, Link JR, Cunningham MR, Rupar MJ, Cunyuzlu PL, Haws TF, Kassam A, Powell F *et al*. 2002 Generation of multiple farnesoid-X-receptor isoforms through the use of alternative promoters. *Gene* **290** 35–43. (https://doi.org/10.1016/S0378-1119(02)00557-7)

Kaeding J, Bouchaert E, Bélanger J, Caron P, Chouinard S, Verreault M, Larouche O, Pelletier G, Staels B, Bélanger A *et al*. 2008 Activators of the farnesoid X receptor negatively regulate androgen glucuronidation in human prostate cancer LNCAP cells. *Biochemical Journal* **410** 245–253. (https://doi.org/10.1042/BJ20071136)

Kalaany NY & Mangelsdorf DJ 2006 LXRS and FXR: the yin and yang of cholesterol and fat metabolism. *Annual Review of Physiology* **68** 159–191. (https://doi.org/10.1146/annurev.physiol.68.033104.152158)

Kiani S, Valizadeh B, Hormazdi B, Samadi H, Najafi T, Samini M & Dehpour AR 2009 Alteration in male reproductive system in experimental cholestasis: roles for opioids and nitric oxide overproduction. *European Journal of Pharmacology* **615** 246–251. (https://doi.org/10.1016/j.ejphar.2009.04.049)

Madeo M, Carrisi C, Iacopetta D, Capobianco L, Cappello AR, Bucci C, Palmieri F, Mazzeo G, Montalto A & Dolce V 2009 Abundant expression and purification of biologically active mitochondrial citrate carrier in baculovirus-infected insect cells. *Journal of Bioenergetics and Biomembranes* **41** 289–297. (https://doi.org/10.1007/s10863-009-9226-6)

Maeda T, Miyata M, Yotsumoto T, Kobayashi D, Nozawa T, Toyama K, Gonzalez FJ, Yamazoe Y & Tamai I 2004 Regulation of drug transporters by the farnesoid X receptor in mice. *Molecular Pharmacology* **1** 281–289. (https://doi.org/10.1021/mp0499656)

Makishima M, Okamoto AY, Repa JJ, Tu H, Learned RM, Luk A, Hull MV, Lustig KD, Mangelsdorf DJ & Shan B 1999 Identification of a nuclear receptor for bile acids. *Science* **284** 1362–1365. (https://doi.org/10.1126/science.284.5418.1362)

Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P *et al*. 1995 The nuclear receptor superfamily: the second decade. *Cell* **83** 835–839. (https://doi.org/10.1016/0092-8674(95)90199-X)

Modica S, Gadaleta RM & Moschetta A 2010 Deciphering the nuclear bile acid receptor FXR paradigm. *Nuclear Receptor Signaling* **8** e005. (https://doi.org/ 10.1621/nrs.08005)

Monte MJ, Marin JJ, Antelo A & Vazquez-Tato J 2009 Bile acids: chemistry, physiology, and pathophysiology. *World Journal of Gastroenterology* **15** 804–816. (https://doi.org/10.3748/wjg.15.804)

Morelli A, Comeglio P, Filippi S, Sarchielli E, Cellai I, Vignozzi L, Yehiely-Cohen R, Maneschi E, Gacci M, Carini M *et al*. 2012 Testosterone and farnesoid X receptor agonist INT-747 counteract high fat diet-induced bladder alterations in a rabbit model of metabolic syndrome. *Journal of Steroid Biochemistry and Molecular Biology* **132** 80–92. (https://doi.org/ 10.1016/j.jsbmb.2012.02.007)

Otte K, Kranz H, Kober I, Thompson P, Hoefer M, Haubold B, Remmel B, Voss H, Kaiser C, Albers M *et al*. 2003 Identification of farnesoid X receptor β as a novel mammalian nuclear receptor sensing lanosterol. *Molecular and Cellular Biology* **23** 864–872. (https://doi.org/10.1128/MCB.23.3.864-872.2003)

Panteghini M, Bonora R & Pagani F 2001 Measurement of pancreatic lipase activity in serum by a kinetic colorimetric assay using a new chromogenic substrate. *Annals of Clinical Biochemistry* **38** 365–370. (https://doi.org/10.1258/0004563011900876)

Parks DJ, Blanchard SG, Bledsoe RK, Chandra G, Consler TG, Kliewer SA, Stimmel JB, Willson TM, Zavacki AM, Moore DD *et al*. 1999 Bile acids: natural ligands for an orphan nuclear receptor. *Science* **284** 1365–1368. (https://doi.org/10.1126/science.284.5418.1365)

Popescu IR, Helleboid-Chapman A, Lucas A, Vandewalle B, Dumont J, Bouchaert E, Derudas B, Kerr-Conte J, Caron S, Pattou F *et al*. 2010 The nuclear receptor FXR is expressed in pancreatic beta-cells and protects human islets from lipotoxicity. *FEBS Letters* **584** 2845–2851. (https://doi.org/10.1016/j.febslet.2010.04.068)

Psychoyos A, Creatsas G, Hassan E, Georgoulias V & Gravanis A 1993 Spermicidal and antiviral properties of cholic acid: contraceptive efficacy of a new vaginal sponge (Protectaid®) containing sodium cholate. *Human Reproduction* **8** 866–869. (https://doi.org/10.1093/oxfordjournals.humrep.a138156)

Rago V, Siciliano L, Aquila S & Carpino A 2006 Detection of estrogen receptors ER-alpha and ER-beta in human ejaculated immature spermatozoa with excess residual cytoplasm. *Reproductive Biology and Endocrinology* **17** 36. (https://doi.org/10.1186/1477-7827-4-36)

Rago V, Aquila S, Panza R & Carpino A 2007 Cytochrome P450arom, androgen and estrogen receptors in pig sperm. *Reproductive Biology and Endocrinology* **6** 23. (https://doi.org/10.1186/1477-7827-5-23)

Rago V, Giordano F, Brunelli E, Zito D, Aquila S & Carpino A 2014 Identification of G protein-coupled estrogen receptor in human and pig spermatozoa. *Journal of Anatomy* **224** 732–736. (https://doi.org/10.1111/joa.12183)

Santoro M, Guido C, De Amicis F, Sisci D, Vizza D, Gervasi S, Carpino A & Aquila S 2013 Sperm metabolism in pig: a role for Peroxisome Proliferator-Activated Receptor (PPAR)gamma. *Experimental Biology* **216** 1085–1092. (https://doi.org/10.1242/jeb.079327)

Scotti E, Gilardi F, Godio C, Gers E, Krneta J, Mitro N, De Fabiani E, Caruso D & Crestani M 2007 Bile acids and their signaling pathways: eclectic regulators of diverse cellular functions. *Cellular and Molecular Life Sciences* **64** 2477–2491. (https://doi.org/10.1007/s00018-007-7280-y)

Sèdes L, Martinot E, Baptissart M, Baron S, Caira F, Beaudoin C & Volle DH 2017 Bile acids and male fertility: from mouse to human? *Molecular Aspects of Medicine* **56** 101–109. (https://doi.org/10.1016/j.mam.2017.05.004)

Seol W, Choi HS & Moore DD 1995 Isolation of proteins that interact specifically with the retinoid X receptor: two novel orphan receptors. *Molecular Endocrinology* **9** 72–85. (https://doi.org/10.1210/mend.9.1.7760852)

Tiangang Li, Francl JM, Boehme S, Ochoa A, Zhang Y, Klaassen CD, Erickson SK & Chiang JYL 2012 Glucose and insulin induction of bile acid synthesis mechanisms and implication in diabetes and obesity. *Journal of Biological Chemistry* **287** 1861–1873. (https://doi.org/10.1074/jbc.M111.305789)

Urner F & Sakkas D 1999*a* Characterization of glycolysis and pentose phosphate pathway activity during sperm entry into the mouse oocyte. *Biology of Reproduction* **60** 973–978. (https://doi.org/10.1095/biolreprod60.4.973)

Urner F & Sakkas D 1999*b* A possible role for the pentose phosphate pathway of spermatozoa in gamete fusion in the mouse. *Biology of Reproduction* **60** 733–739. (https://doi.org/10.1095/biolreprod60.3.733)

Urner F, Leppens-Luisier G & Sakkas D 2001 Protein tyrosine phosphorylation in sperm during gamete interaction in the mouse: the influence of glucose. *Biology of Reproduction* **64** 1350–1357. (https://doi.org/10.1095/biolreprod64.5.1350)

Vega A, Martinot E, Baptissart M, De Haze A, Vaz F, Kulik W, Damon-Soubeyrand C, Baron S, Caira F & Volle DH 2015 Bile acid alters male mouse fertility in metabolic syndrome context. *PLoS ONE* **10** e0139946. (https://doi.org/10.1371/journal.pone.0139946)

Vignozzi L, Morelli A, Filippi S, Comeglio P, Chavalmane AK, Marchetta M, Toce M, Yehiely-Cohen R, Vannelli GB, Adorini L *et al*. 2011 Farnesoid X receptor activation improves erectile function in animal models of metabolic syndrome and diabetes. *Journal of Sexual Medicine* **8** 57–77. (https://doi.org/10.1111/j.1743-6109.2010.02073.x)

Vlahcevic ZR, Heuman DM & Hylemon PB 1991 Regulation of bile acid synthesis. *Hepatology* **13** 590–600. (https://doi.org/10.1002/hep.1840130331)

Wang H, Chen J, Hollister K, Sowers LC & Forman BM 1999 Endogenous bile acids are ligands for the nuclear receptor FXR/BAR. *Molecular Cell* **3** 543–553. (https://doi.org/10.1016/S1097-2765(00)80348-2)

Watanabe M, Houten SM, Mataki C, Christoffolete MA, Kim BW, Sato H, Messaddeq N, Harney JW, Ezaki O, Kodama T *et al*. 2006 Bile acids induce energy expenditure by promoting intracellular thyroid hormone activation. *Nature* **439** 484–489. (https://doi.org/10.1038/nature04330)

World Health Organization 2010 *WHO Laboratory Manual for the Examination and Processing of Human Semen*, 5th ed. UK: Cambridge University Press.

Yamazaki M, Miyake M, Sato H, Masutomi N, Tsutsui N, Adam KP, Alexander DC, Lawton KA, Milburn MV, Ryals JA *et al*. 2013 Perturbation of bile acid homeostasis is an early pathogenesis event of drug induced liver injury in rats. *Toxicology and Applied Pharmacology* **268** 79–89. (https://doi.org/10.1016/j.taap.2013.01.018)

Zara V, Dolce V, Capobianco L, Ferramosca A, Papatheodorou P, Rassow J & Palmieri F 2007 Biogenesis of eel liver citrate carrier (CIC): negative charges can substitute for positive charges in the presequence. *Journal of Molecular Biology* **365** 958–967. (https://doi.org/10.1016/j.jmb.2006.10.077)

Zhang Y 2010 Farnesoid X receptor-acting through bile acids to treat metabolic disorders. *Drugs Future* **35** 635–642. (https://doi.org/10.1358/dof.2010.035.08.1520865)

Zhang Y, Kast-Woelbern HR & Edwards PA 2003 Natural structural variants of the nuclear receptor farnesoid X receptor affect transcriptional activation. *Journal of Biological Chemistry* **278** 104–110. (https://doi.org/10.1074/jbc.M209505200)