Human sperm anatomy and endocrinology in varicocele: role of androgen receptor

Carmela Guido1,2,4,*, Marta Santoro1,2,*, Francesca De Amicis1,3, Ida Perrotta4, Salvatore Panza1,4, Vittoria Rago4, Maria Grazia Cesario1,3, Marilena Lanzino1,3,† and Saveria Aquila1,3,†

1Centro Sanitario, 2Post-graduate School in Clinical Pathology, 3Department of Pharmacy and Sciences of Health and Nutrition and 4Department of Biology, Ecology and Earth Science (Di.B.E.S.T.), University of Calabria, Cosenza, Arcavacata di Rende 87030, Italy

Correspondence should be addressed to S Aquila who is now at Department of Pharmacy and Sciences of Health and Nutrition, University of Calabria, Cosenza, Arcavacata di Rende (CS) 87036, Italy; Email: aquisav@libero.it

*(C Guido and M Santoro contributed equally to this work)
†(M Lanzino and S Aquila are joint senior authors)

Abstract

The study of androgens involved in male reproduction has been object of intense efforts, while their reported action on human male gametes is limited. We previously described the presence of androgen receptor (AR) in sperm with a role related to the modulation of the PI3K pathway. In the present study, we investigated the expression of AR and its ultrastructural location in normal sperm as well as in spermatozoa obtained from varicocele patients. We observed a reduced AR content in varicocele sperm with respect to healthy sperm by western blot analysis and transmission electron microscopy (TEM). The ultrastructural location of AR was detected mainly on the head membrane as well as in the nucleus, neck, and mitochondria. Influence of dihydrotestosterone (DHT) treatment on cholesterol efflux was increased in normal sperm, while it was reduced or absent in varicocele sperm. To better understand DHT/AR significance in human male gametes, we evaluated triglyceride content and lipase, acyl-CoA dehydrogenase, and glucose-6-phosphate dehydrogenase activities upon DHT treatment. The metabolic outcome glimpsed in normal sperm was an increased metabolic rate, while ‘varicocele’ sperm economized energy. Taken together, our results reveal DHT and AR as new players in sperm endocrinology, indicating that varicocele sperm may have difficulty in switching to the capacitated status. A decreased AR expression and a consequent reduced responsiveness to DHT in sperm may represent molecular mechanisms involved in the pathophysiology of varicocele leading to male infertility. This study revealed new detrimental effects of varicocele on sperm at the molecular level.

Reproduction (2014) 147 589–598

Introduction

Androgens, which are mainly produced in testicular Leydig cells, are essential throughout male life, as they play a fundamental role in sexual differentiation, maintenance of spermatogenesis, and expression of secondary sexual characteristics (Wang et al. 2009). The action of androgens is mediated by androgen receptor (AR), a member of the nuclear receptor superfamily, which functions as a ligand-inducible transcription factor (Wang et al. 2009). Naturally occurring AR ligands are testosterone and its more active metabolite dihydrotestosterone (DHT). Activated AR may trigger either genomic or rapid, nongenomic signaling pathways, and a crosstalk between the two AR functional pathways has also been proposed (Bonaccorsi et al. 2008, Grosse et al. 2012). Several findings indicate androgens and AR to be fundamental actors in the regulation of normal spermatogenesis and fertility. The function of AR has been shown to be required for the completion of meiosis and the transition of spermatocytes to haploid round spermatids, and the replacement of androgens alone is able to initiate qualitatively complete spermatogenesis in gonadotropin-deficient mice (Singh et al. 1995, De Gendt et al. 2004). Conversely, AR gene inactivation severely disrupts spermatogenesis by interrupting meiosis completion, thereby abolishing the mature sperm production and leading to male sterility (Walters et al. 2010). Nevertheless, mechanisms by which androgens regulate male fertility still need to be clarified and are the subject of intense efforts. Indeed, there are few reports on the role of androgens in the ejaculated sperm physiology. Ejaculated mammalian spermatozoa express AR, playing a role in the modulation of sperm survival and capacitation by the regulation of the PI3K/AKT signaling pathway (Aquila et al. 2007, Zalata et al. 2013). However, the presence and function of AR in
spermatozoa are not universally accepted. It has been shown that male total Ar knockout (T-Ar⁻/⁻) mice exhibit incomplete germ cell development and lowered serum testosterone levels, which result in azoospermia and infertility (Zhang et al. 2006). It has also been reported that the deletion of Ar gene in mouse germ cells does not affect spermato genesis and male fertility (Tsai et al. 2006). Besides, the consequences of AR loss in particular types of testicular cells remain unclear.

Ejaculated mammalian spermatozoa are fascinating, highly differentiated cells characterized by extremely polarized cellular architecture and function. During their lifespan, spermatozoa are exposed to different physiological conditions. In the male genital tract, they remain in a quiescent state accumulating and/or economizing energy substrates (uncapacitated spermatozoa), while in the female genital tract, they undergo a functional maturation process, known as capacitation, acquiring the capability to fertilize an oocyte (Suarez 2008). Capacitation is a complicated process regulated by different molecules and several signaling pathways and involves many physiological changes including increased metabolic rate and energy expenditure (Blackmore et al. 1990, Baldi et al. 2000, De Amicis et al. 2012a, 2012b, Perrotta et al. 2012, Aquila et al. 2013). Different hormones, such as insulin and leptin, as well as sex steroids, namely estrogens and progesterone, have been reported to affect testicular function (Guido et al. 2012) and human sperm metabolism (De Amicis et al. 2011, Guido et al. 2011). In normal androgen-responsive tissues in vivo, androgens stimulate lipogenic gene expression (Heemers et al. 2003). Furthermore, they are widely regarded as important anabolic agents in the muscle or with lipolytic effects in adipose tissue (Xu et al. 1990, Elashoff et al. 1991). A role for androgens/AR in the management of energy metabolism in spermatozoa has not been addressed yet.

In the present study, to gain insight into the biological significance of the DHT/AR pathway in human male gametes, we investigated the expression of AR and its ultrastructural location in normal sperm as well as in spermatozoa obtained from varicocele patients. The influence of DHT/AR on capacitation and lipid and glucose metabolism was also evaluated.

Materials and methods

Chemicals

Percoll (colloidal PVP-coated silica for cell separation), sodium bicarbonate, sodium lactate, sodium pyruvate, dimethyl sulfoxide (DMSO), Earle’s balanced salt solution (EBSS), DHT, goat polyclonal actin antibody (Ab), monoclonal mouse anti-AR Ab, and all other chemicals were purchased from Sigma Chemical Industries. Casodex (Cax), a specific antagonist of AR, was from AstraZeneca. 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 ECL, and HEPES sodium salt were from Amersham Pharmacia Biotech. EBSS without calcium, without magnesium, without phenol red, and without NaHCO₃ (uncapacitating medium) was from Genaxxon Bioscience (Milan, Italy). Colloidal gold-conjugated goat anti-mouse IgG secondary Ab was from Sigma–Aldrich, and peroxidase-coupled anti-mouse, anti-rabbit, and anti-goat IgG secondary Abs were from Santa Cruz Biotechnology. Cholesterol oxidase (CHOD)–peroxidase (POD) enzymatic colorimetric kit, triglyceride assay kit, lipase activity kit, and glucose-6-phosphate dehydrogenase (G6PDH) activity assay kit were from Inter-Medical (Biogemina Italia Srl, Catania, Italy). DHT was dissolved in ethanol (EtOH), while Cax in DMSO, and the experiments carried out with the vehicles alone did not reveal differences between treated and untreated samples (data not shown).

Semen samples and spermatozoa preparations

Human semen samples were collected, according to the World Health Organization (WHO)-recommended procedure, from healthy normozoospermic volunteer donors of proven fertility. Briefly, semen samples with normal parameters of volume, sperm count, motility, morphology, and vitality, according to the WHO Laboratory Manual (WHO 2010), were used in this study. Samples from patients affected by varicocele who consulted us for fertility investigation were also used in the study. Spermatozoa preparations were obtained as described previously (De Amicis et al. 2011). Reflux of blood in the pampiniform plexus was determined by palpation employing the Valsalva maneuver. Physical examination is the reference standard to diagnose varicoceles in subfertile men. Additional radiological imaging is not necessary to diagnose subclinical varicocele, because only a varicocele detected by physical examination should be considered potentially significant (Pryor & Howards 1987). Varicocele samples used in this study were from patients with diagnosed varicocele of grade III (visible without palpation) on the left testis, and their ejaculates were found to have total sperm count of 16×10⁶ sperm cells per ejaculate, percentage of motility of 32%, percentage of normally formed features of 27%, and percentage of viability of 60%. The study was approved by the local medical–ethical committee, and all participants gave their informed consent.

Processing and treatments of ejaculated sperm

Each sperm sample was obtained by pooling three (normozoospermic) or four (varicocele) ejaculates of different subjects. In our experience, this was necessary to obtain enough cells to carry out all the tests (Aquila et al. 2007, De Amicis et al. 2012a, 2012b). The final sperm concentration of the resuspended samples during treatments was 20×10⁶/ml. Each assay was carried out at least three times using at least three different sperm samples. Therefore, we evaluated at least a total of nine different normozoospermic specimens and 12 different varicocele specimens.

The samples were then subjected to centrifugation (800 g) on a discontinuous Percoll density gradient (80:40% v:v) (Guido et al. 2011). The 80% Percoll fraction was examined using an optical
microscope equipped with a 100× oil objective to ensure that a pure sample containing only spermatozoa was obtained. These sperm had a motility of about 65% and a viability of 80% in both normal and pathological samples. An independent observer inspected several fields for each slide. Particularly, the same number of Percoll-purified sperm from both normal and pathological samples were washed with unsupplemented Earle’s medium and incubated for 30 min at 37 °C and 5% CO₂, without (control, NC) or with the following treatments: increasing DHT concentrations (0.1, 1, and 10 nM) according to previous studies (De Amicis et al. 2013, 2012a, 2012b). Percoll-purified sperm samples, washed twice by centrifugation, were incubated in the presence of 10 μM Cax alone or combined with 1 nM DHT. At the end of the sperm incubation period, culture medium was recovered by centrifugation, lyophilized, and subsequently dissolved in 1 ml of the reaction buffer. The samples were incubated for 10 min at room temperature, and then the cholesterol content was measured at 505 nm. Cholesterol content results are presented as mg per 10×10⁶ spermatozoa as reported in our previous studies (De Gendt et al. 2004, Wang et al. 2009).

**Immunogold labeling for AR**

Immunogold labeling assay was carried out as reported previously (De Amicis et al. 2013). Briefly, sperm fixed overnight in 4% paraformaldehyde were washed in PBS to remove excess fixative, dehydrated in graded alcohol, infiltrated in LR White resin, and polymerized in a vacuum oven at 45 °C for 48 h, while 60 nm ultrathin sections were cut and placed on coated nickel grids for post-embedding immunogold labeling with the rabbit polyclonal Ab against human AR. Potential nonspecific labeling was blocked by incubating the sections in PBS containing 5% normal goat serum, 5% BSA, and 0.1% cold-water fish gelatine at room temperature for 1 h. The sections were then incubated overnight at 4 °C with mouse polyclonal AR Ab at a dilution of 1:500 in PBS buffer. The sections were then incubated in 10 nm colloidal gold-conjugated anti-mouse IgG secondary Ab at a dilution of 1:50 for 2 h at room temperature. The sections were then washed in PBS, fixed in glutaraldehyde, counterstained with uranyl acetate, and examined under a Zeiss EM 900 transmission electron microscope. To assess the specificity of immunolabeling, negative control assays were carried out for the corresponding sections of sperm that were labeled with colloidal gold-conjugated secondary Ab with normal mouse serum instead of the primary Ab.

**Western blot analysis of sperm proteins**

Percoll-purified sperm samples, washed twice by incubating medium, were incubated as described above and then centrifuged for 5 min at 5000 g. The pellet was resuspended in lysis buffer as described previously (Aquila et al. 2013). An equal amount of protein (80 μg) was boiled for 5 min, separated on an 11% PAGE, transferred onto nitrocellulose membranes, and probed with an appropriate dilution of the indicated primary Ab. The binding of the secondary Ab was revealed with the ECL Plus Western blotting detection system, according to the manufacturer’s instructions. Western blot analysis was carried out in at least four independent experiments, and more representative results are presented.

**Measurement of cholesterol content in the sperm culture medium**

Cholesterol content in the culture medium in which human spermatozoa were incubated was measured in duplicate using a CHOD–POD enzymatic colorimetric method according to the manufacturer’s instructions, as described previously (De Gendt et al. 2004). Percoll-purified sperm samples, washed twice with uncapacitating medium, were incubated for 30 min at 37 °C and 5% CO₂ in the same medium (control) or in the presence of increasing DHT concentrations. Other samples were incubated in the presence of 10 μM Cax alone or combined with 1 nM DHT. At the end of the sperm incubation period, culture medium was recovered by centrifugation, lyophilized, and subsequently dissolved in 1 ml of the reaction buffer. The samples were incubated for 10 min at room temperature, and then the cholesetril content was measured at 505 nm. Cholesterol content results are presented as mg per 10×10⁶ spermatozoa as reported in our previous studies (De Gendt et al. 2004, Wang et al. 2009).

**Lipase activity assay**

Lipase activity was evaluated as reported previously using the method of Panteghini et al. (2001) based on the use of 1,2-o-dilauryl-rac-glycero-3-glutaric acid-6′-methylresorufin) ester (DGGR) as substrate. Sperm extracts weighing 50 μg were loaded into individual cuvettes containing a buffer for spectrophotometric determination (Panteghini et al. 2001). DGGR is cleaved by lipase, resulting in an unstable dicarboxylic acid ester, which is spontaneously hydrolyzed 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 lipase activity in the sample. The estimated reference interval was 6–38 U/l (μmol/min per mg protein). Enzymatic activity was determined with three control media: one without the substrate, another without the coenzyme (colipase), and one without either the substrate or the coenzyme (data not shown).

**Acyl-CoA dehydrogenase activity assay**

Acyl-CoA dehydrogenases are a class of enzymes that catalyze the initial step in each cycle of fatty acid β-oxidation in the mitochondria of cells. Acyl-CoA dehydrogenase activity assay was carried out on sperm samples using a modification of the method described by Lehman et al. (1990) and
De Amicis et al. (2011). In brief, after cell lysis, 70 µg of sperm proteins were added to the buffer containing 20 mM MOPS (3-(N-morpholino)propanesulfonic acid), 0.5 mM EDTA, and 100 mM FAD at pH 7.2. The reduction of FAD to FADH was read at 340 nm upon the addition of octanoyl-CoA (100 µM) every 20 s for 1.5 min. Data are expressed as nmol/min per mg protein. Enzymatic activity was determined with three control media: one without octanoyl-CoA as the substrate, another without the coenzyme (FAD), and one without either the substrate or the coenzyme (data not shown).

G6PDH activity assay

The conversion of NADP to NADPH, catalyzed by G6PDH, was measured by the increase in absorbance at 340 nm (De Amicis et al. 2011). Sperm samples, washed twice with uncapacitating medium, were incubated in the same medium (control) for 30 min at 37 °C and 5% CO2. Other samples were incubated in the presence of the treatment agents at the indicated concentrations. After incubation, 50 µl of sperm extracts were loaded into individual cuvettes containing a buffer (100 mM triethanolamine, 100 mM MgCl2, 10 mg/ml glucose-6-phosphate, and 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 per 10^6 sperm. Enzymatic activity was determined with three control media: one without glucose-6-phosphate as the substrate, another without the coenzyme (NADP+), and the last one without either the substrate or the coenzyme (data not shown).

Statistical analysis

Transmission electron microscopy (TEM) was performed in at least three independent experiments, and statistical analysis was carried out by counting the numbers of gold particles over cross-sectional profiles of 80 sperm from both normozoospermic (healthy control) and varicocele samples, and the difference between the means was calculated using Student’s t-test. Western blot analysis was carried out in at least four independent experiments. Data obtained from cholesterol assay, triglyceride assay, and lipase activity, acyl-CoA dehydrogenase activity, G6PDH activity assays (ten replicate experiments using duplicate determinations) are presented as means ± S.E.M. Differences in mean values were calculated using ANOVA with a significance level of P ≤ 0.05. The Wilcoxon test was used after ANOVA as a post hoc test.

Results

AR is differentially expressed in the spermatozoa of healthy or varicocele-affected subjects

The expression of AR protein in the ejaculated sperm of healthy or varicocele-affected subjects was investigated by western blot analysis using a MAB raised against the AR epitope mapping at the 299–316 aa in the N-terminus region of AR of human origin. According to our previous study (Aquila et al. 2007), two protein bands with molecular weights of 110 and 87 kDa respectively were detected. The mainly expressed isoform was the 87 kDa form, corresponding to AR-A (Fig. 1). Interestingly, AR content was strongly reduced in ‘varicocele’ samples (Fig. 1), suggesting a role for AR in varicocele pathophysiology.

Figure 1 Reduction of AR expression in varicocele sperm. N1, N2, and N3 expression of AR in ejaculated sperm from three different samples of normal men and V1, V2, and V3 expression of AR in ejaculated sperm from three different samples of varicocele-affected men. The number on the left corresponds to molecular masses of the marker proteins. β-actin was used as loading control. The experiments were repeated at least four times, and the autoradiographs are the results of one representative experiment.

De Amicis et al. (2011). In brief, after cell lysis, 70 µg of sperm proteins were added to the buffer containing 20 mM MOPS (3-(N-morpholino)propanesulfonic acid), 0.5 mM EDTA, and 100 µM FAD at pH 7.2. The reduction of FAD to FADH was read at 340 nm upon the addition of octanoyl-CoA (100 µM) every 20 s for 1.5 min. Data are expressed as nmol/min per mg protein. Enzymatic activity was determined with three control media: one without octanoyl-CoA as the substrate, another without the coenzyme (FAD), and one without either the substrate or the coenzyme (data not shown).

G6PDH activity assay

The conversion of NADP to NADPH, catalyzed by G6PDH, was measured by the increase in absorbance at 340 nm (De Amicis et al. 2011). Sperm samples, washed twice with uncapacitating medium, were incubated in the same medium (control) for 30 min at 37 °C and 5% CO2. Other samples were incubated in the presence of the treatment agents at the indicated concentrations. After incubation, 50 µl of sperm extracts were loaded into individual cuvettes containing a buffer (100 mM triethanolamine, 100 mM MgCl2, 10 mg/ml glucose-6-phosphate, and 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 per 10^6 sperm. Enzymatic activity was determined with three control media: one without glucose-6-phosphate as the substrate, another without the coenzyme (NADP+), and the last one without either the substrate or the coenzyme (data not shown).

Statistical analysis

Transmission electron microscopy (TEM) was performed in at least three independent experiments, and statistical analysis was carried out by counting the numbers of gold particles over cross-sectional profiles of 80 sperm from both normozoospermic (healthy control) and varicocele samples, and the difference between the means was calculated using Student’s t-test. Western blot analysis was carried out in at least four independent experiments. Data obtained from cholesterol assay, triglyceride assay, and lipase activity, acyl-CoA dehydrogenase activity, G6PDH activity assays (ten replicate experiments using duplicate determinations) are presented as means ± S.E.M. Differences in mean values were calculated using ANOVA with a significance level of P ≤ 0.05. The Wilcoxon test was used after ANOVA as a post hoc test.

Results

AR is differentially expressed in the spermatozoa of healthy or varicocele-affected subjects

The expression of AR protein in the ejaculated sperm of healthy or varicocele-affected subjects was investigated by western blot analysis using a MAB raised against the AR epitope mapping at the 299–316 aa in the N-terminus region of AR of human origin. According to our previous study (Aquila et al. 2007), two protein bands with molecular weights of 110 and 87 kDa respectively were detected. The mainly expressed isoform was the 87 kDa form, corresponding to AR-A (Fig. 1). Interestingly, AR content was strongly reduced in ‘varicocele’ samples (Fig. 1), suggesting a role for AR in varicocele pathophysiology.
Specifically, the expression of the short AR variant was reduced in varicocele samples, whereas the expression of the long variant remained unaffected.

**Immunogold localization of AR in human sperm**

Ultrastructural analysis of spermatozoa by TEM was carried out to determine the localization of AR in human sperm. As shown in Fig. 2, in healthy samples, the label was present mostly within the head, decorating the plasma membrane as well as the nucleus (Fig. 2A and B). Appreciable evidence of gold particles was also observed in the neck and the mitochondria-containing midpiece sperm compartments (Fig. 2D and E). On the contrary, AR was only faintly present as a component of the sperm flagellum, between the ribs of the fibrous sheath, outer dense fibers, and axoneme (Fig. 3A, B, D, and E).

Negative control experiments with normal rabbit serum did not reveal any label in the corresponding sperm regions (Figs 2C and F and 3C and F). According to the above-described western blot data, in ‘varicocele’ samples, a reduction in the number of gold particles was evident in the head (Fig. 4A and B), along the neck and lower down the midpiece (Figs 4D and E and 5A and B). Moreover, labeling was absent in the tail of sperm in varicocele samples (Fig. 5D and E). Negative control experiments with normal mouse serum did not reveal any signal in the corresponding sperm regions (Figs 4C and F and 5C and F). Statistical elaboration of labeling density as the numbers of gold particles over the cross-sectional profiles of sperm from both normozoospermic and varicocele samples carried out with Student’s t-test showed a significance of $P<0.0001$ (data not shown).

**Figure 3** AR ultrastructural location in the midpiece and tail region of sperm of normozoospermic subjects. Sperm samples were collected and prepared as described in the Materials and methods section. Micrographs of the sections of ejaculated sperm of normozoospermic subjects probed with mouse MAB against human AR: (A and B) longitudinal and cross sections of the midpiece; (D and E) longitudinal and cross sections of the tail region; and (C and F) results of the negative control (N) experiments carried out on the corresponding sections of sperm where normal mouse serum instead of the primary Ab was used. Results are representative of three similar experiments.

**Figure 4** AR ultrastructural location in the head and neck of sperm of varicocele patients. Sperm samples were collected and prepared as described in the Materials and methods section. Micrographs of the sections of ejaculated sperm of varicocele patients probed with mouse MAB against human AR: (A and B) sperm head; (D and E) neck and midpiece; and (C and F) results of the negative control (N) experiments carried out on the corresponding sections of sperm where normal mouse serum instead of the primary Ab was used. Results are representative of three similar experiments.
DHT induces cholesterol efflux in human sperm

Our previous study has demonstrated the involvement of AR in capacitation (Aquila et al. 2007); therefore, to better understand its role in fertilization potential, we examined the effects of DHT/AR on cholesterol efflux, using three different normozoospermic samples as well as three different varicocele samples. Treatment with DHT (at concentrations ranging from 0.1 to 10 nM) increased cholesterol efflux with a maximum effect at 1 nM compared with the untreated samples (NC, Fig. 6). Co-treatment with 10 μM Cax, a specific AR antagonist, abrogated this DHT action, indicating that it is mediated by AR. In varicocele samples, under control conditions, cholesterol efflux was remarkably lower than that in the normal samples. DHT at a concentration of 1 nM was able to induce cholesterol efflux, although to a lesser extent than that in the normozoospermic samples.

DHT increases G6PDH activity in human sperm

Glucose is metabolized through glycolysis and the pentose phosphate pathway (PPP) in spermatozoa, the latter producing NADPH to achieve fertilization (Urner & Sakkas 1999). The possible role of DHT in this context was investigated by evaluating the activity of G6PDH, the key rate-limiting enzyme in the PPP. In healthy samples, DHT was able to greatly induce G6PDH enzymatic activity, and 10 μM Cax reverted the effect of 1 nM DHT. On the contrary, treatment with DHT, at all the tested concentrations, was unable to affect G6PDH activity in varicocele samples (Fig. 7).

DHT interferes with lipid metabolism in human sperm

Testosterone is a fat-reducing hormone that promotes lipolysis and reduces fatty acid synthesis (De Pergola 2000). As a role for this steroid in lipid metabolism in sperm is yet to be defined, we evaluated intracellular triglyceride content upon treatment with increasing DHT concentrations. As shown in Fig. 8A, DHT was able to decrease triglyceride content in a dose-dependent manner, and the combination of Cax with 1 nM DHT reverted the effect. On the contrary, in varicocele samples, we observed an increase in triglyceride content. To determine the mechanism through which DHT alters sperm lipid metabolism, we evaluated its action on lipase and acyl-CoA.

Figure 5 AR ultrastructural location in the midpiece and tail region of sperm of varicocele patients. Sperm samples were collected and prepared as described in the Materials and methods section. Micrographs of the sections of ejaculated sperm of varicocele patients probed with mouse MAB against human AR: (A and B) longitudinal and cross sections of the midpiece; (D and E) longitudinal and cross sections of the tail region; and (C and F) results of the negative control (N) experiments carried out on the corresponding sections of sperm where normal mouse serum instead of the primary Ab was used. Results are representative of three similar experiments.

Figure 6 Influence of DHT on cholesterol efflux in human sperm. Purified spermatozoa (normal and varicocele affected) were incubated in unsupplemented Earle’s medium for 30 min at 37 °C and 5% CO2, in the absence (NC) or in the presence of treatment agents at the indicated concentrations. Cholesterol content in the culture medium in which human ejaculated spermatozoa were incubated was measured using an enzymatic colorimetric assay. Columns represent means ± S.E.M. of ten independent experiments carried out in duplicate. Data are expressed as mg/107 sperm. *P<0.05 vs control and **P<0.02 vs control.
dehydrogenase activities. Interestingly, lipase activity was induced upon DHT treatment, with the maximum effect being observed at the concentration of 1 nM, while using 10 \( \mu \text{M} \) Cax, the 1 nM DHT-induced action was revoked (Fig. 8B). Concomitantly in normal samples, DHT at all the tested concentrations induced acyl-CoA dehydrogenase activity, and the combination of Cax with 1 nM DHT abolished the effect (Fig. 8C). In varicocele samples, DHT was effective at the concentration of 1 nM producing a significant induction reverted by 10 \( \mu \text{M} \) Cax co-treatment.

Discussion

To date, varicocele represents one of the most common causes of male infertility, and although this pathology has been studied extensively, the mechanisms through which it can influence male fertility are not fully defined. Although it is well known that androgens are involved in the development of male sexual characteristics as well as in spermatogenesis, their action in the context of male infertility is not fully clarified. In the present study, we investigated the anatomical location of AR and its possible action on energy management in sperm at the ultrastructural level. Our data strengthen the functional role of AR in human sperm outcomes and underline the molecular basis of male infertility in human varicocele.

The presence of AR has been shown previously by immunofluorescence in the head and in the midpiece, corresponding to the site of the mitochondria, in human sperm (Solakidi et al. 2005, Aquila et al. 2007). In this study, by TEM with immunogold labeling analysis, we analyzed the anatomical regions containing AR in ‘healthy’ human sperm. Interestingly, numerous gold particles decorated the head at both the membrane and the nucleus. Labeled regions corresponding to the connecting piece (neck), especially in the segmented columns, were observed. Elevated AR expression was detected in the mitochondria, whereas its expression was lower in the connecting piece.

**Figure 7** Effects of DHT on glucose metabolism in human sperm. Washed spermatozoa (normal and varicocele affected) were incubated in unsupplemented Earle’s medium for 30 min at 37 °C and 5% CO\(_2\), in the absence (NC) or in the presence of treatment agents at the indicated concentrations. G6PDH activity assay was carried out as described in the Materials and methods section. Columns represent means ± S.E.M. *\( P < 0.005 \) vs control and **\( P < 0.001 \) vs control.

**Figure 8** Effects of DHT on lipid metabolism in human sperm. Washed spermatozoa were incubated in unsupplemented Earle’s medium for 30 min at 37 °C and 5% CO\(_2\), in the absence (NC) or in the presence of treatment agents at the indicated concentrations. (A) Triglyceride assay was carried out as described in the Materials and methods section. Columns represent means ± S.E.M. *\( P < 0.05 \) vs control and **\( P < 0.01 \) vs control. (B) Lipase activity assay was carried out as described in the Materials and methods section. Columns represent means ± S.E.M. *\( P < 0.05 \) vs control and **\( P < 0.01 \) vs control. (C) Octanoyl-CoA dehydrogenase activity assay was carried out as described in the Materials and methods section. Columns represent means ± S.E.M. *\( P = 0.05 \) vs control; **\( P < 0.01 \) vs control; and ***\( P < 0.005 \) vs control.
was slowly reduced along the flagellum to the end piece. It is worth noting that ultrastructural analysis revealed that the expression of AR in varicocele samples was decreased. Particularly, rare gold particles were detected on the entire sperm from the head to the tail region, demonstrating that low levels of AR are present in this pathological condition. These data are consistent with the results of the western blot analysis, where a reduced expression of both AR isoforms was observed, and with recent data reported by Zalata et al. (2013).

A functional role for steroid receptors in capacitation, acrosome reaction, motility, and survival in sperm has been reported (Adeoya-Osiguwa et al. 2003). It is well known that human ejaculated sperm are not capable of fertilizing oocyte as they need two important sequential steps: capacitation and acrosome reaction. During capacitation, sperm undergo final maturation by increasing cholesterol efflux, which in turn alters sperm membrane fluidity, tyrosine phosphorylation of sperm proteins, and metabolic rate.

In this study, we demonstrated that DHT through AR is able to induce a capacitation marker such as cholesterol efflux, accordingly with an increase in protein phosphorylation reported previously (Aquila et al. 2007). The importance of androgens in the completion of male gamete maturation process during epididymal transit has been proved by the presence of AR in epididymal tissues (Zhou et al. 2002). Therefore, our data sustain the concept that AR influences sperm capacitation together with estrogen receptor (ER) and progesterone receptor (PR) (Guido et al. 2011, De Amicis et al. 2012a, 2012b). Interestingly, basal cholesterol efflux was reduced in varicocele sperm with respect to healthy sperm, and the effect of DHT was abated in the former. This result may probably be due to the reduced expression of AR determining a decreased responsiveness to DHT. Besides, our data are in agreement with the idea that grade II/III infertile varicocele presents an alteration in sperm plasma membrane dynamics due a decreased cholesterol mobility (Buffone et al. 2006).

The presence of AR in sperm mitochondria allowed us to hypothesize that this receptor could be involved in energy metabolism. It has been demonstrated that testosterone deficiency leads to increased fat deposition, whereas high testosterone levels inhibit adipocyte development, promoting lipolysis and reducing fatty acid synthesis (Salam et al. 2012). In normal sperm, DHT was able to reduce triglyceride content, while it induced lipase and acyl-CoA dehydrogenase activities, suggesting a lipolytic effect. Capacitated sperm imply

---

**Figure 9** Roles of AR in sperm. DHT goes across the sperm membrane and binds to AR inducing its phosphorylation and different effects. (A) Glucose enters into sperm via glucose transporter (Glut), and it is phosphorylated to glucose-6-phosphate (Gluc-6-P) to be used in the PPP and in glycolysis, producing NADPH and ATP respectively. Upon DHT treatment, AR induces the activity of G6PDH, the first enzyme of the PPP, contributing to the regulation of glucose metabolism in the sperm; (B) AR upon DHT treatment is strictly involved in the PI3K/AKT pathway in the regulation of sperm survival; (C) DHT/AR signaling is able to regulate lipid metabolism inducing lipase and octanoyl-CoA dehydrogenase activities, thus reducing triglyceride levels; and (D) AR upon DHT treatment induces cholesterol efflux and tyrosine phosphorylation of sperm proteins, both closely related to and representing the main events of the capacitation process. During capacitation, an increase in membrane fluidity occurs, and cholesterol escapes from the cell after the activation of PKA that induces tyrosine phosphorylation of sperm proteins.
increased metabolism and overall energy expenditure, and previous studies have demonstrated that hormones induce capacitation together with energy expenditure (Aquila et al. 2009, Goodson et al. 2012).

Therefore, we retain our previous finding that DHT interacting with AR may activate the receptor through phosphorylation (Aquila et al. 2007), increasing metabolic activities and fueling capacitation in sperm. Interestingly, we observed a reduced response to DHT in varicocele sperm; therefore, it could be speculated that varicocele sperm are affected by a dismetabolic syndrome due to inefficacious enzymatic activities leading to the accumulation of triglycerides. Accordingly, a strong association of testosterone with lipogenesis has been observed at the systemic level during dismetabolic syndrome (Salam et al. 2012). Although the regulation of sperm energy metabolism is not well known, it may be generalized that uncapacitated sperm are associable with an anabolic metabolism, while capacitation with a catabolic metabolism. In this context, our data on varicocele sperm might indicate that they have difficulty in switching to the capacitated status.

It has been found that testosterone can modulate metabolic enzymes through a rapid nongenomic action in rat hepatocytes and also acts on G6PDH activity in fish (Baque et al. 1996, Sunny et al. 2002). Consistently, in normal sperm, we observed that DHT induced G6PDH activity, while the effect did not occur in varicocele sperm. Previous data from our laboratory have widely confirmed that nuclear receptors in sperm mediate functional maturation by regulating their metabolic status (De Amicis et al. 2012a, 2012b, Santoro et al. 2013). In this study, we defined the role of AR in healthy and varicocele samples, supporting the importance of the balance of steroids/receptors in human sperm physiology. The reduction of AR expression and responsiveness to DHT in varicocele sperm allowed us to define AR as a key player in the functional maturation of human male gametes. In Fig. 9, we present the proposed roles for the AR in sperm.

In conclusion, varicocele affects testicular function in a variety of ways including spermatogenesis, semen quality, sperm functions, and morphology. From our data, it emerges that this pathology induces damage in the male gamete at the molecular level, opening a new chapter in the already multifactorial pathophysiology of the varicocele.

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.

Funding
This study was supported by Ministero dell’Istruzione, dell’Università e della Ricerca (MIUR EX-60%-2013).

AR expression in healthy and varicocele sperm

Acknowledgements
The authors cordially thank Dr Vincenzo Cunisolo (Biogemina Italia Srl, Catania, Italy) for the technical and scientific assistance. They also thank Perrotta Enrico for the excellent technical assistance and Serena and Maria Clelia Gervasi for the English language review of the manuscript.

References


Received 24 October 2013
First decision 10 December 2013
Revised manuscript received 19 December 2013
Accepted 15 January 2014