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Method

Mass spectrometry profiling of oxysterols in human sperm identifies 25-hydroxycholesterol as a marker of sperm function

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ABSTRACT

Cholesterol is a main lipid component of sperm cell that is essential for sperm membrane fluidity, capacitation, and acrosomal reaction. Recent data obtained in bovine sperm showed that sperm capacitation is associated to the formation of oxysterols, oxidized products of cholesterol. The aim of this study was to profile oxysterol content in human semen, and to investigate their potential role in sperm pathophysiology. Among the 12 oxysterols analyzed, 25-hydroxycholesterol (25-HC) resulted the most represented in normozoospermic samples, and its concentration positively correlated with spermatozoa number. We detected Cholesterol 25-hydroxylase, the enzyme responsible for 25-HC production, in human spermatozoa at the level of the neck and the post acrosomal area. Upon incubation with spermatozoa, 25-HC induced calcium and cholesterol transients in connection with the acrosomal reaction. Our results support a role for 25-HC in sperm function.

1. Introduction

Spermatozoa are immotile and unable to fertilize an oocyte at the stage of releasing from the seminiferous epithelium. Fertilizing capacity of sperm cells is acquired during their passage through the epididymis [1]. Cholesterol, a main component of cell membrane system, affects sperm membrane fluidity, and promotes the complex mechanism leading to the evolution of a capacitated state, which is ultimately completed in the female reproductive tract. Cholesterol excreted from the epididymal epithelium contributes to the maturation of transiting sperm [2], and cholesterol content in sperm membrane is tight regulated during epididymal transit [1].

Capacitation is a process consisting of several steps leading to changes in sperm motility and acrosomal responsiveness; these events are highly dependent upon changes in plasma membrane cholesterol [3]. One of the key steps of capacitation is the loss of cholesterol from the sperm plasma membrane [4] in a process that can be promoted by albumin and bicarbonate, which facilitate lipoprotein-mediated cholesterol efflux [5–7].

De Lamirande and Gagnon [8] highlighted the role of reactive oxygen species (ROS) concentration in sperm capacitation. Mild ROS formation would favor signaling events that are involved in sperm capacitation [9,10]; on the other hand, higher rates of ROS formation would result in irreversible deterioration of sperm cell function [11]. Concerning ROS-mediated mechanisms in sperm function, most attention has been reserved to phospholipid peroxidation [12]. Little attention has been devoted to cholesterol by-products generated by autoxidation, i.e. oxysterols, notwithstanding cholesterol is the most abundant lipid component of sperm cells, and is an excellent sensor of lipid peroxidation [13]. In addition, cholesterol can be transformed enzymatically into oxysterols that collectively are recognized as potent bioactive molecules [13]. Recently, Brouwers et al. [14] reported on the presence and formation of oxysterols in bovine sperm, and showed the formation of oxysterols during capacitation. These results represent a significant advance in our understanding of the redox regulation of sperm capacitation, opening new perspectives of study on the mechan-

Abbreviations: CTC, chlortetracycline; ROS, reactive oxygen species; BHT, butylated hydroxytoluene; 7α-HC, 7α-hydroxycholesterol; 7β-HC, 7β-hydroxycholesterol; 7KC, 7-Ketocholesterol; 4β-HC, 4β-hydroxycholesterol; 4α-HC, 4α-hydroxycholesterol; 5α, 6α-EC, 5α,6α-epoxycholesterol; 5β, 6β-EC, 5β,6β-epoxycholesterol; ,5α,6β-triol, Triol, cholestane-3β; 6-OCDO, 6-oxo-cholestan-3β,5α-diol; 25-HC, 25-hydroxycholesterol; and 27-HC, 27-hydroxycholesterol; Ch25H, Cholesterol-25-hydroxylase

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isms that regulate the male fertility physiology. Since oxysterols are more hydrophilic than cholesterol, they move more freely out of the membrane and may account for most of the actions currently attributed to cholesterol.

The main objective of the present study was to identify and quantify, for the first time, the different species of oxysterols in human semen from normozoospermic, oligoasthenoteratozoospermic and asthenozoospermic patients. The secondary aim was to investigate the potential role of oxysterols in sperm pathophysiology.

2. Material and methods

2.1. Study population and design

To investigate the profile of human sperm oxysterols in subjects with normal and altered sperm characteristics, we recruited 150 consecutive subjects referring at the Centre of Andrology of S.M. Goretti Hospital (Latina) for analysis of seminal fluid between January 2012 and June 2014. Was performed a clinical history to collect personal information including lifestyle factors, sexual and reproductive status and medical history. Eligible patients were men, 18–50 years-old. Exclusion criteria included past medical history of epididymoorchitis, prostatitis, genital trauma, testicular torsion, urinary tract infection, presence of other genital endocrine or andrologic diseases. None of the subjects had been treated medically or surgically in the 3 months prior to the study. In order to investigate the possible correlation to oxidative stress and semen oxysterols profile we included a group of patients with varicocele given the demonstrated relationship between varicocele and oxidative stress [15,16]. Varicocele was diagnosed by scrotal color-doppler ultrasound during rest and Valsalva maneuver. According to physical examination, the varicocele was graded as grade ≥II. Semen examination was performed in all patients.

The study procedure was developed according to the guidelines of the referring Ethic Committee, which approved the protocol, and the Helsinki Declaration of 1975. The study was registered at clinicaltrials.gov with identifier # NCT02062229.

2.2. Semen analysis

Semen samples were collected by masturbation after 3–5 days of sexual abstinence and analyzed after liquefaction for 60 min at 37 °C. Semen analysis was assessed by light microscope according to World Health Organization guidelines (WHO, 2010). Informed consent to the processing of data was obtained from all patients included in the study. The following variables were taken into consideration: ejaculate volume (mL), total sperm number (x 10^6/ejaculate), progressive motility (%), and morphology (% abnormal forms). Nine semen samples containing $>1\times10^6$ leukocytes/mL were excluded because leukocytes are recognized as another major source of ROS in semen [17]. Moreover we excluded from our study seven azoospermic samples. Therefore we investigated 134 semen samples from the same number of patients, aged 34.5 ± 7.5. Patients were classified according to the sperm parameters: Group 1≥50th percentile (33 normozoospermic men), Group 2≤5th percentile (32 oligoasthenoteratozoospermic men), Group 3 only progressive motility ≤5th percentile (25 asthenozoospermic men) and Group 4 (44 patients with varicocele). An aliquot of semen were stored at −80 °C until GC-MS analysis.

2.3. GC–MS analysis

Oxysterols were determined by GC–MS using deuterium-labeled internal standards as described by Dzeletovic et al. and Iuliano et al. [18,19]. In brief, for the oxysterols analysis was used 1 mL of semen and were added 10 μL BHT in ethanol (5 mg/mL), 50 μL EDTA (10 mg/mL) and 10 μL of ethanol containing deuterium labeled internal standards. Alkaline hydrolysis was performed on the samples for 2 h at room temperature with stirring. Then sterols were extracted in chloroform: methanol (2:1,v/v). Solvent was evaporated under a stream of nitrogen, the sample was dissolved in 1 mL of toluene. Oxysterols were separated from cholesterol by solid phase extraction (silica cartridges 100 mg). The solvent was evaporated under a stream of nitrogen and after samples were converted to trimethylsilyl ethers by treatment with 130 μL Sylon HTP (hexamethyldisilylazane:trimethylchlorosilane:pyridine, 3:1:9) (Supelco, Bellafonte, PA) at 60 °C for 30 min. After incubation, the solution was evaporated under a stream of nitrogen, and the residue dissolved in n-hexane and transferred to an autosampler vial. Analyses were performed on an Agilent 6890N GC equipped with a 7683 series automatic liquid sampler, and interfaced with an Agilent 5973 Mass Spectrometer (Agilent Technologies; Palo Alto, CA). Separation was carried out on a 30 m capillary column (HP-5MS 30 m 0.25 mm ID, 0.25 μm thickness). Quantification of oxysterols was made by the isotope dilution method.

2.4. Western blot analysis

To demonstrate the presence of Cholesterol-25-hydroxylase (Ch25H) in human spermatozoa we used Western blot analysis in 3 different normozoospermic patients. To separate spermatozoa, we performed a density gradient using the whole semen sample. The gradient was made up of the following layers/gradients 55%, 80%, and 100% (SupraSperm, Origio, Denmark). The semen was gently stratified on top of the discontinuous gradient and centrifuged for 25 min at 800g; the seminal plasma was discarded and spermatozoa were collected from the lower gradient (100% layer), resuspended in 2 mL PBS, and centrifuged at 800g for 10 min. Spermatozoa were lysed with Ripa Buffer (Tris HCl pH 8 50 mM, NaCl 130 mM, EDTA 1 mM, Triton X-100 1%, SDS 0.1%, Sodium Deoxycholate 0.1%) mixed with protease inhibitor cocktail (Clontech, Cat.#635673). Protein concentration were determined using Bio-Rad Protein Assay (Bio-Rad Cat.#112792). Equal amounts of protein samples (80 μg/well) were separated electrophoretically by a 12% SDS-PAGE and transferred to PVDF membranes (Amersham Hybond-P). The membranes were blocked for 1 h in T-TBS with 5% Bovine non-fat dry milk. After membranes were incubated with primary antibody against Ch25H (Abcam, Cambridge, UK) 1:250, and against α-tubulin (Abcam, Cambridge, UK) 1:50000 as control, overnight at 4 °C. Then the blots were incubated with anti-Mouse HRP antibody (1:10000) and visualized using WesternBright ECL Kit (Advasta K-12045-D20). As positive control for Ch25H detection we used THP-1 cell line (ATCC TIB-202).

2.5. Immunofluorescence assay

To determine the localization of Ch25H, we performed immunofluorescence assay of three normozoospermic patients. Sperm cells were recovered from density gradient, as described above, and were rinsed with PBS Buffer. Cells (6×10^5) were cytospun for 5 min at 100 x g onto a glass slide. Cells spot was fixed in PFA 4% for 15 min at RT. After the spots were incubated with PBS-NH_4Cl 50 mM to quench PFA autofluorescence and permealized with Triton X100 0.5%. Cells were blocked with PBS-gelatin 0.2% for 45 min and incubated with primary antibody against Ch25H (1:50) in PBS-gelatin 0.2% o/n at 4 °C in a wet chamber. After cells were incubated with anti-mouse 488 Alexafluor secondary antibody (1:500) for 45 min at RT in the dark. Nuclei were counterstained with DAPI (1:1000) for 5 min. To exclude unspecific fluorescence signal, negative control experiments were carried out using the secondary antibody in the absence of the antibody against cholesterol 25-hydroxylase. As positive control for Ch25H detection we used THP-1 cell line (ATCC TIB-202), as they are known to express the enzyme. The slides were examined under a fluorescence microscope (Leica DM4000 B) with 1000X magnification.