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Comparison of Methods for Rapid Determination of Cholesterol Concentration in Human Sperm Membrane in Clinical Laboratory Practice

A. G. Mironova[a,b,*], S. I. Afanasyeva[c], A. V. Sybachin[d], V. V. Spiridonov[d], M. A. Bolshakov[e], and E. Yu. Simonenko[c]

[a] *Human Reproduction Clinic "Altravita" (LLC "IVF CENTER"), Moscow, 117186 Russia*
[b] *N.M. Emanuel Institute of Biochemical Physics, Russian Academy of Sciences, Moscow, 119334 Russia*
[c] *Lomonosov Moscow State University, Faculty of Physics, Department of Biophysics, Moscow, 119991 Russia*
[d] *Lomonosov Moscow State University, Faculty of Chemistry, Department of High Molecular Compounds, Moscow, 119234 Russia*
[e] *FSBI "Federal Research Center "Pushchino Scientific Center for Biological Research of the Russian Academy of Sciences", Pushchino, 119991 Russia*
**e-mail: agm90@mail.ru*

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Abstract—Objective: Cholesterol is an important structural component of the plasma membrane of mammalian cells. Cholesterol, among other important roles, plays a special role in sperm membranes. Change in the lipid composition of the sperm membrane, particularly the outflow of cholesterol, is an integral part of the process of capacitation and subsequent acrosomal reaction necessary for the sperm to fertilize an egg. Deviations in cholesterol concentration in sperm membrane may indicate a decrease in the fertilizing potential of sperm. To determine the optimal method for rapid analysis of the cholesterol content in human sperm membranes in the IVF laboratory, four methods of quantitative determination of cholesterol were compared in terms of practicality and effectiveness of their use to assess the concentration of cholesterol in human sperm membranes: the method of enzymatic colorimetric detection, the Lieberman–Burchard method, infrared spectroscopy and high-performance liquid chromatography. **Methods:** 101 ejaculates of patients with established normozoospermia (according to WHO criteria) were used in the work. Spermatozoa were separated from the semen by double centrifugation with the addition of DPBS medium. The resulting cellular pellet was used to determine the concentration of cholesterol in a sample by one of four methods: enzymatic colorimetric detection (FCD), HPLC, Lieberman-Burchard method, infrared spectroscopy. **Results and Discussion:** The following cholesterol concentrations were obtained by enzymatic colorimetric detection, Lieberman–Burchard, infrared spectroscopy and high-performance liquid chromatography: 1.0 ± 0.3, 1.32 ± 0.15, 5.1 ± 1.8 and 1.53 ± 0.18 nmol/10^6 cells, respectively. The Lieberman–Burchard method, enzymatic colorimetric detection and HPLC showed similar results, the obtained average cholesterol concentrations coincide within the error. The mean cholesterol concentration in sperm membranes obtained using infrared spectroscopy method significantly exceeds the values presented in the literature and the values obtained using other methods. In addition, this method requires an amount of analyzed material that significantly exceeds the volume of one ejaculate. **Conclusions:** As a result of comparing four methods of quantitative cholesterol analysis, the method of enzymatic colorimetric detection is proposed as a method of rapid analysis of cholesterol in human sperm membranes suitable for routine use in a clinical laboratory. The advantages of this method include the low toxicity of the method, it's cost-effectiveness and a significant reduction in the time of complete analysis: from sample preparation to obtaining the result.

Keywords: cholesterol, sperm, membrane, chromatography, infrared spectroscopy, Lieberman–Burkhard reaction

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INTRODUCTION

Cholesterol is an important structural component of the plasma membrane of mammalian cells [1]. The structural formula of the cholesterol molecule is shown in Fig. 1. Cholesterol is oriented in the phospholipid bilayer in a way that its polar hydroxyl group faces the aqueous phase [2], and the hydrophobic steroid ring is oriented parallel to the fatty acid residues [3–6]. As a result of the interaction of the cholesterol ring system with the fatty acid residues of neighboring phospholipids, the degree of ordering of the bilayer increases, and a liquid

Abbreviations: ECD, Enzymatic colorimetric detection.

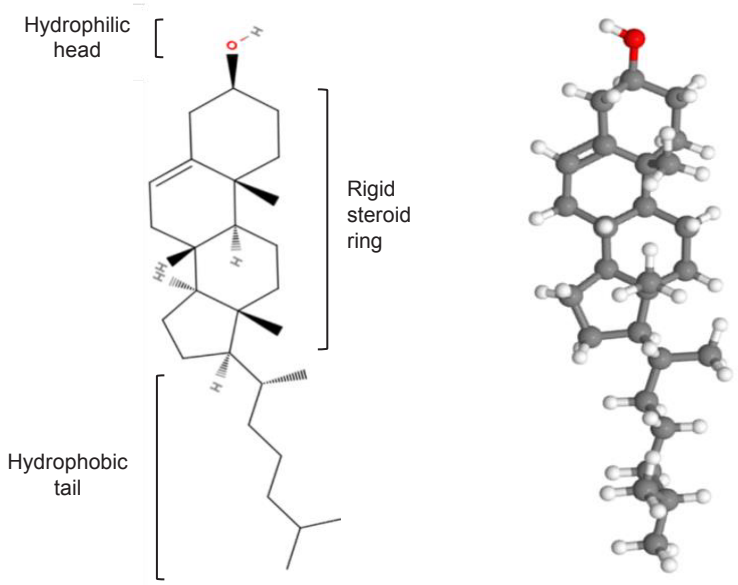

Fig. 1. Structure of the cholesterol molecule. All four rings of the sterol group have *trans*-conformations making the cholesterol molecule flat. The double bond between the fifth and sixth carbon atoms of the chain provides rigidity to the cholesterol molecule [2]. For the formation of an idea of the three-dimensional structure of cholesterol, it is important to note that the OH group, two methyl groups and a side chain are located on one side of the ring skeleton (β-configuration) [1]. The hydroxyl group in the cholesterol molecule imparts an amphiphilic character to the compound and contributes to the orientation of the cholesterol molecule in the bilayer.

crystalline ordered phase L_o is formed [7, 8]. In the L_o-phase, lipids exhibit a lateral diffusion coefficient like that measured in "liquid" phospholipid bilayers [9], while the area per phospholipid decreases and the thickness of the lipid bilayer increases [10–12], and the membrane becomes more condensed [13]. This leads to a change in the permeability [14] and mechanical properties of the membrane [15].

At low cholesterol concentrations (<10 mol %), the dipalmitoyl phosphatidylcholine/cholesterol model bilayer undergoes a gel-to-liquid crystal phase transition, as observed for pure lipid bilayers, and the phase transition temperature T_m slightly shifts towards higher temperatures. At high cholesterol concentrations (>30 mol %) t he main phase transition is suppressed, while at intermediate concentrations (10–30 mol %) most studies report the coexistence of two phases: disordered liquid crystalline L_d and ordered liquid crystal L_o (above the phase transition temperature) or solid crystal S_o and L_o (below the phase transition temperature) [2, 3].

In addition to interacting with membrane phospholipids, cholesterol can also modulate the properties and functions of membrane proteins [16–18]. A variety of integral membrane proteins, including ion channels, membrane receptors, and enzymes, are sensitive to changes in the surrounding lipid bilayer [19–22]. Some proteins also bind directly to cholesterol, resulting in their activation or inactivation [23, 24]. The activity of cholesterol-regulated proteins is likely mediated through sterol-sensing domains [25]. The normal functioning of proteins included in lipid rafts also depends on cholesterol, since it is necessary for their formation [26].

In sperm membranes, cholesterol, among other functions, has a special role [27]. Changes in the lipid composition of the sperm membrane, particularly the efflux of cholesterol, are an integral part of the capacitation process and the subsequent acrosomal reaction required by the sperm to fertilize the egg [28, 29]. Deviations in cholesterol concentration from the norm may indicate a decrease in the fertilizing potential of spermatozoa [30].

A method that allows for the accurate quantitative determination of cholesterol in the range of nanomolar concentrations is required to determine the concentration

of cholesterol in human spermatozoa under clinical conditions.

In this study, four methods for the quantitative determination of cholesterol were compared based on their practicality and effectiveness in assessing the concentration of cholesterol in human sperm membranes: the enzymatic colorimetric detection method, the Liebermann–Burchard method, infrared (IR) spectroscopy, and high-performance liquid chromatography. The aim of this study was to select the optimal method for rapid analysis of cholesterol content in human sperm membranes in an IVF laboratory.

RESULTS AND DISCUSSION

Preparation of Samples for Determination of Cholesterol Concentration in Sperm Membranes

In this study, 101 ejaculates from patients with normozoospermia were used to determine the concentration of cholesterol in sperm membranes. Spermatozoa were washed from seminal plasma for subsequent determination of cholesterol by one of four methods: enzymatic colorimetric detection (ECD) method—24 ejaculate samples, HPLC—14 ejaculate samples, Liebermann–Burchard method—21 ejaculate samples, IR spectroscopy—42 ejaculate samples. The IR spectroscopy method required the pooling of several ejaculates (~6) to determine the concentration of cholesterol in sperm membranes, thus 7 samples were analyzed by this method.

Determination of Cholesterol Concentration in Sperm Membrane by Enzymatic Colorimetric Detection (ECD)

Enzymatic assay methods are commonly used to determine cholesterol levels in blood plasma. The protocol of the ECD method was modified to determine the cholesterol concentration in sperm membranes. To determine the concentration of cholesterol in sperm membranes, cholesterol was extracted from sperm membranes using Triton X-100.

As a result of the study of 24 patients' ejaculates, the following average value of cholesterol concentration in sperm membranes was obtained:

$$C_{ENZ} = 1 \pm 0.3 \text{ nmol/mln.}$$

The main advantages of enzymatic methods include the absence of the need to work with aggressive chemical reagents and the exceptional specificity of enzymes, which significantly reduces the impact of external components on the analytical reaction. This makes enzymatic methods good candidates for routine use within the IVF laboratory.

Determination of Cholesterol Concentration by the Indirect Liebermann–Burchard Method

The concentration of cholesterol was determined by a chemical indirect (extraction) method with cholesterol extraction using the Folch method, followed by photometric determination of the reaction products of cholesterol with the Liebermann–Burchard reagent.

Using this method, 21 samples of patient ejaculate were analyzed. The amount of cholesterol in the sample was determined using a calibration graph. The following average value of cholesterol concentration C_{LB} was obtained for the sample:

$$C_{LB} = 1.32 \pm 0.15 \text{ nmol/mln.}$$

The obtained result is consistent with literature data [31, 32]. However, despite the accuracy of the obtained results, this method requires complex sample preparation using aggressive volatile reagents: concentrated sulfuric acid, acetic anhydride, methanol, and chloroform. This method is not suitable as a routine method for determining cholesterol concentration in a clinical laboratory, it can only be used in a specially equipped chemical laboratory, but it can be recommended as a reference method for the calibration and detection of errors in other methods and test systems.

Determination of Cholesterol Concentration by HPLC

The cholesterol molecule has expressed hydrophobic properties, which allows to perform quantitative analysis using reversed-phase chromatography. In this study, an HPLC with a UV detector was used. The amount of

cholesterol in the samples was determined by the peak area at T_R = 8.4 relative to the calibration curve with known concentrations.

The mass of cholesterol in the sample was determined using a calibration graph, after which the average cholesterol concentration was calculated using the formula:

$$C_{HPLC} = \frac{m_{chol}}{M_{chol} \times N_{sperm}}, \quad (1)$$

where C_{HPLC}—cholesterol concentration in the sample, nmol/mln; m_{chol}—mass of cholesterol in the sample, μg; M_{chol}—molar mass of cholesterol, mg/mol; N_{sperm}—number of spermatozoa in sample, mln.

As a result of the analysis of 14 samples of patient ejaculates, the following average value of cholesterol concentration in human spermatozoa was obtained:

C_{HPLC} = 1.53 ± 0.18 nmol/mln.

Determination of Cholesterol Concentration by IR Spectroscopy

The cholesterol molecule contains the following fragments that can be identified by IR spectroscopy: a hydroxyl group, a double C=C bond, and CH_2-groups in the system of condensed cyclic fragments. The absorption band at 1646 cm^{-1} is the most selective for determining the amount of cholesterol, corresponding to the vibrations of the double C=C bond of the cyclohexene group. This method required samples in which the amount of cholesterol significantly exceeded physiological values. For each sample, it was necessary to prepare ~20 mg of dried sperm sediment, which corresponds to the collection and processing of ~6 ejaculates. Even with this amount of sample, the absorption band at 1646 cm^{-1} was poorly expressed. Among the absorption bands observed in the IR spectrum, the band at 2940 cm^{-1} had the highest intensity, corresponding to the excitation of oscillations of the CH_2-groups in the system of condensed cyclic fragments, relative to which further calculations were carried out.

The mass of cholesterol in the sample was determined using a calibration graph, after which the concentration of cholesterol in the sample was calculated using the formula:

$$C_{IR} = \frac{m_{chol}}{M_{chol} \times N_{sperm}}, \quad (2)$$

where C_{IR}—concentration of cholesterol in the sample, nmol/mln; m_{chol}—mass of cholesterol in the sample, mg; M_{chol}—molar mass of cholesterol, mg/mol; N_{sperm}—number of spermatozoa in sample, mln.

Using this method, seven samples were analyzed and the following average value for cholesterol concentration in human sperm was obtained:

C_{IR} = of 5.1 ± 1.8 nmol/mln.

The obtained result significantly exceeds the values presented in the literature [31, 32] and the data obtained using other methods. The discrepancy in the results was primarily due to the non-strict selectivity of the absorption band at 2940 cm^{-1}, corresponding to the excitation of oscillations of the CH_2-groups that are present not only in cholesterol but also in all membrane lipids.

The average concentrations of cholesterol in human sperm membranes obtained using the ECD, Lieberman–Burchard, HPLC, and IR spectroscopy methods are presented in Table 1.

The presented data indicate that the results obtained by the Liebermann–Burchard, HPLC and enzymatic analysis methods correlate with the literature data [31, 32]. The mean value of cholesterol concentration

Table 1. Mean cholesterol concentrations (nmol/mln) in human spermatozoa obtained by different methods and their comparison with literature data [31, 32]

Mean cholesterol concentration, nmol/mln					
HPLC	enzymatic analysis	IR spectroscopy	Lieberman–Burkhard method	literature data [31]	literature data [32]
1.53 ± 0.18	1.0 ± 0.3	5.1 ± 1.8	1.32 ± 0.15	1.04 ± 0.12	1.2 ± 0.3

in spermatozoa obtained by the ECD method was lower than the values obtained using the Liebermann–Burchard method and HPLC. This may be due to the increased resistance of lipid rafts, which contain predominantly cholesterol in cell membranes, to the effects of detergents and, therefore, incomplete extraction of cholesterol. The average value of cholesterol concentration in sperm membranes obtained using IR spectroscopy significantly exceeded the cholesterol concentration values obtained using other methods and differed significantly from the values presented in the literature.

EXPERIMENTAL

Materials and methods. Reagents. Reagent kit for cholesterol determination Cholesterol-ECD (Agat, Russia); Dulbecco's solution with calcium and magnesium (DPBS, PanEco, Russia); cholesterol, acetic anhydride (Sigma, the United States); chloroform (reagent grade), methanol (reagent grade), sulfuric acid (special purity grade) (Reakhim, Russia); potassium bromide for spectroscopy (Aladdin, China).

Sperm samples. In this study, 101 ejaculates of patients with established normozoospermia (according to WHO criteria) were used. Written informed consent was obtained from all participants prior to inclusion in the study. The inclusion criteria for the study were the following indicators: sperm concentration in the ejaculate—not less than 50 million/mL; the proportion of progressively motile spermatozoa of $a + b$ categories — not less than 32%; morphology—not less than 4% in accordance with Kruger's strict criteria; concentration of leukocytes in the ejaculate—not more than 0.1 mln/mL.

Sample preparation. DPBS solution was added to the ejaculate in a ratio of 1 : 2 (*v*/*v*), centrifuged at 300 g for 10 min, the supernatant was collected, the sediment was resuspended in 2 mL DBPS and centrifuged at 300 g for 10 min. The supernatant was collected, the sediment was resuspended in 200–500 μL DPBS, depending on the volume of sediment, the concentration of cells was calculated in the Makler Counting Chamber (the count was performed at least 5 times in different aliquots, and the average value of sperm concentration in the sample was calculated). The cholesterol concentration in the sample was then determined using one of the methods described below.

Determination of cholesterol concentration using the enzymatic colorimetric method. To 100 μL of the cell suspension, 100 μL of a 2% solution of Triton X100 in DPBS was added, incubated for 1 h at 37°C, after which 2 mL of the enzyme-chromogenic mixture Cholesterol ECD (cholesterol esterase, cholesterol oxidase, peroxidase, aminoantipyrine, sodium hydroxybenzenesulfonate, sodium cholate, phosphate buffer). For the preparation of the calibration sample, 2 mL of enzyme-chromogenic mixture were added to a standard cholesterol solution (51.7 nmol cholesterol in a 200 μL sample). Samples were thoroughly mixed by vortexing and incubated for 25 min at 25°C in the dark. At the end of the incubation, the spermatozoa were precipitated by centrifugation at 300 g for 10 min. The supernatant was collected to record the absorption spectrum on a spectrophotometer PE-5400UF (EKROSKHIM, Russia) in in the wavelength range of 300–700 nm with a step of 1 nm in cuvettes with an optical path length of 10 mm. The calculation of the cholesterol concentration in the analyzed sample was performed using the formula:

$$C = \frac{E_0}{Ec}\frac{51.7}{n}, \quad (3)$$

where C—cholesterol concentration in the test sample, nmol/mln; E_0—optical density of the test sample at a wavelength of 514 nm; E_C—optical density of the calibration sample at a wavelength of 514 nm; 51.7—amount of cholesterol in the calibration sample, nmol; n—number of spermatozoa in the sample, mln.

Lipid extraction using the Folch method. Lipid extraction was performed according to the Folch method [33]. 4 mL of chloroform-methanol solution (2 : 1) were added to 100 μL of cell suspension. 1, *v*/*v*) was vortexed for 5 min; 750 μL of water was added and centrifuged at 10,000 g for 15 min at room temperature; the lower organic layer containing lipids was collected and the extraction procedure was repeated (adding 4.3 mL chloroform, 700 μL methanol and 500 μL water, vortexing for 5 min; centrifugation at 10000 g for 15 min); the organic layer was collected and combined with the sample ob-

tained in the first stage; the samples were dried in a Laborota-4000 rotary evaporator (Heidolph, Germany).

Quantitative determination of cholesterol using the Liebermann–Burchard method. Dried lipid extracts were re-dissolved in 500 μL of Liebermann–Burchard reagent (chloroform/acetic anhydride/sulfuric acid 3 : 2 : 0.1, *v*/*v*/*v*). For the construction of a calibration curve, 500 μL of Liebermann–Burchard reagent were added to a series of standard cholesterol solutions (20–200 nmol cholesterol in a 10 μL sample). Samples were incubated for 20 min at 25°C in the dark. Absorption spectra were recorded using a PE-5400UF spectrophotometer (EKROSKHIM, Russia) in the wavelength range of 300–700 nm with a step of 1 nm in cuvettes with an optical path length of 10 mm. The characteristic absorption spectrum for the Liebermann–Burchard reaction has two absorption maxima at 410 and 650 nm. For the construction of a calibration graph and further determine the concentration of cholesterol in the samples, the optical density value at a wavelength of 650 nm was used.

High-performance liquid chromatography (HPLC) method. Dried lipid extracts were re-dissolved in 500 μL of mobile phase (isopropanol/acetonitrile/water 60 : 30 : 10, *v*/*v*/*v*). Chromatographic determination of cholesterol content was performed using a 1100 HPLC system (Agilent; the United States) using a Zorbax XDB-C18 4.6 reversed phase column. × 150 mm × 5 μm (Agilent, the United States). Conditions of chromatographic separation: temperature – 28°C, eluent A: isopropanol/acetonitrile/water (60 : 30 : 10, *v*/*v*/*v*), flow rate 1 mL/min, spectrophotometric detector DAD. The absorption spectrum was obtained at a wavelength of 205 nm, 40 μL of sample was applied to the column. The total analysis time was 14 min. Cholesterol was identified by retention time on the column using Agilent software (the United States). For the construction of a calibration curve, chromatograms were recorded for a series of cholesterol solutions (10–100 μg in a 40 μL isopropanol/acetonitrile/water sample). The calibration curve was constructed based on the peak area of the spectrum observed at TR = 8.4 min, which corresponds to the retention time of cholesterol.

IR spectroscopy method. The cell sediment, obtained earlier after centrifugation, was placed in a drying oven at 60°C for 48 h until the liquid had completely evaporated. Samples for recording IR spectra were prepared using the suspension method in potassium bromide. On an analytical balance, 200 mg of pre-ground spectrally pure potassium bromide was weighed and placed in a porcelain cup. For the formation of the final sample, dried cells from several patients were used, which were combined to achieve a total sample weight of ~20 mg. The dried cells were thoroughly ground in the same porcelain cup, the contents of the cup were thoroughly mixed, completely transferred into a press mold and pressed, and as a result, a transparent or translucent tablet was obtained. The IR spectrum of the obtained tablet was recorded using a Specord M80 spectrometer (Carl Zeiss, Germany) in frequency range 400–4000 cm^{-1}. For the construction of a calibration curve, IR spectra were recorded for a series of cholesterol samples (1.1–4.7 mg cholesterol in 200 mg potassium bromide). The calibration graph was constructed based on the height of the spectrum peak at 2940 cm^{-1}.

CONCLUSIONS

A comparison of four methods for quantitative analysis of cholesterol in the membrane of human spermatozoa was carried out. It has been shown that the IR spectroscopy method is unsuitable for determining the cholesterol concentration in the spermatozoa of an individual patient or sperm donor, since this method requires an amount of analyzed material significantly exceeding the volume of a single ejaculate.

The Liebermann–Burchard, enzymatic colorimetric detection and HPLC methods showed similar results, the obtained average values of cholesterol concentration coincide within the error limits. At the same time, the Liebermann–Burchard and HPLC methods require more complex sample preparation and the use of aggressive reagents for lipid extraction, which also excludes the possibility of their use as methods for assessing cholesterol concentration within the IVF laboratory, but these methods can be used as reference methods.

The ECD method has several significant advantages as a routine laboratory method for determining the concentration of cholesterol in sperm membranes compared to the Liebermann–Burchard and HPLC methods. First, this is the low toxicity of the method, as well as the lower cost of reagents and a significant reduction in the time of the complete analysis: from sample preparation to obtaining the result.

The ECD method can be recommended as a method for express analysis of cholesterol concentration in sperm membranes under IVF laboratory conditions.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. They were also approved by the Ethics Committee of the Altravita Human Reproduction Clinic, LLC IVF CENTER. Informed consent was obtained from all individual participants involved in the study.

CONFLICT OF INTEREST

No conflicts of interest was declared by the authors.

AUTHOR CONTRIBUTION

All authors contributed equally to the writing of the article.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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