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PHOSPHOLIPID COMPOSITION OF HUMAN SPERM AND SEMINAL PLASMA IN RELATION TO SPERM FERTILITY

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The phospholipid and fatty acid composition of sperm was studied in 8 healthy and 16 infertile men. Infertile men randomly formed from the patients with normal semen parameters according to WHO criterion. Therefore, all semen parameters of infertile patients were similar to the same characteristics of the semen of healthy men, except the abnormal forms. The amount of abnormal forms in infertile men was significantly higher than in healthy men. Sperm from infertile men show a drastic loss of phosphatidyl ethanolamine. At the same time, the significant increase of phosphatidyl serine in the sperm and seminal plasma of sterile patients was found. Lysophosphatidyl serine in the sperm of the infertile men was detected. Fatty acid composition of the semen of infertile men was altered. The levels of stearic and *n*-3 polyunsaturated fatty acids (eicosopentaenoic and docosahexaenoic acids) was dramatically lowered, but the values of some *n*-6 polyunsaturated fatty acids (linolenic and docosatetraenoic) acids increased. There was significant positive correlation between docosahexaenoic acid and sperm motility ($r = .82$, $p < .001$) and negative correlation between linolenic acid and spermatozoa motility ($r = –0.58$, $p < .05$). Infertility of men with normal semen quality can originate from the disorder of sperm lipid metabolism. The drastic loss of phosphatidyl ethanolamine and *n*-3 polyunsaturated fatty acids with simultaneous enhancement of phosphatidyl serine and some *n*-6 polyunsaturated fatty acids in sperm could be an important cause of male infertility.

Keywords fatty acids, fertility, phospholipid, seminal plasma, human sperm

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Lipids play an important role in the functional activity of sperm and thus in male infertility [9]. Semen analysis of mammals has shown that processing, capacitation and acrosome reaction are associated with multiple specific modifications of phospholipid composition of sperm plasma membrane [3, 16–18]. Asthenozoospermia is associated with lower concentration of sperm docosahexaenoic acid [27] and higher omega6/omega3 fatty acids ratio [7]. Oligozoospermia and azoospermia are linked to alteration of phospholipid and neutral lipid composition [9, 19]. Unfortunately, the sperm lipid composition of infertile men with normozoospermia is poorly understood. Some authors did not find a strong correlation between abnormal sperm morphology and male infertility [6]. Many investigators have shown that even subnormal motile densities and morphology did not predict the infertile male [10]. It seems that decreased sperm fertile potential can be associated with alteration of sperm lipid content.

The present investigation was undertaken to determine the phospholipid and fatty acid composition of human normozoospermic semen in relation to its fertility.

MATERIALS AND METHODS

Semen samples were obtained by masturbation from 8 normal healthy men (22–46 years of age) who had children and 16 infertile patients (23–50 years of age) with semen characteristics similar to normal ones. However, the abnormal forms of sperm in this group was elevated (Table 1).

Sperm Count and Separation

Sperm were counted immediately after liquefaction of semen according to the WHO protocol [26] with some modifications. For evaluation of the number of sperm, 2 counter chambers were used. Dissolved native semen was placed in one chamber. Another portion of the sperm were immobilized by treating with the solution of sulfuric acid. Quantity of immotile sperm in both chambers was compared. The difference between them show the number of motile sperm. Dead sperm and abnormal cells were determined after staining by eosine and nigrosine (Table 1). Sperm and seminal plasma were separated by centrifugation of the semen at 600 g for 12 min at room temperature. Semen was previously diluted by 3 volumes of Krebs–Ringer phosphate buffer. The supernatant was aspirated and used for lipid extraction. Sperm pellets were washed twice by the same solution. The pellet was resuspended in 0.85% NaCl to receive the

Table 1. The semen profile of healthy and infertile men (mean ± SE)

Semen volume (mL)	Sperm count ($\times 10^6$/mL)	Total sperm count in whole ejaculate ($\times 10^6$)	Motile cells ($\times 10^6$/mL)	Dead cells ($\times 10^6$/mL)	Abnormal cells ($\times 10^6$/mL)
		Healthy men (n = 8)			
4.0 ± 0.4	103 ± 15	413 ± 67	68 ± 9	31 ± 7	26 ± 6
		Infertile men (n = 16)			
4.2 ± 0.4	103 ± 9	415 ± 47	52 ± 7	42 ± 6	43 ± 5

*$p < .05$, compared to healthy men.

suspension, which contained 10–50 × 10^6 sperm/mL. This suspension then was homogenized and used for lipid extraction.

Lipid Analysis

Extraction of lipids was performed within 120 min after semen collection. The lipids were extracted with methanol–chloroform (2:1 v/v) according to Bligh and Dyer [1]. The ratio of water–methanol–chloroform was close to 0.8:2:1 in the monophasic system and 0.9:1:1 in the biphasic system. The lower phase, which contained purified lipid, was aspirated and then dried on a rotary evaporator. The upper phase was extracted once more in the same manner. The lower phase was combined with the first extract and dried on the rotary evaporator. For more complete extraction of anionic phospholipids, the procedure recommended by Palmer was used [16]. The lipid extract was stored in a small volume of chloroform at –20ºC.

Phospholipids were analyzed by two-dimensional, high-resolution, micro thin-layer chromatography (HRMTLC) on silica gel KSK-2 (Russia) by using chloroform–methanol–benzene–ammonium (28%) (65:30:10:6, by vol) and chloroform–methanol–benzene–acetone–acetic acid–water (70:30:10:4:5:1, by vol) [25] as solvent systems. For phospholipid development we used 60 × 60-mm^2 plates. To determine the quantity of the phospholipid phosphorus, the molybdate spray reagent was used [23]. This method permitted us to analyze the phospholipid composition of individual samples of semen.

Individual phospholipids were revealed with molybdate [23] and malachite green reagents [24]. The anthrone reagent [22] was also used to determine phospholipid, sulfolipid, and glycolipid containing spots, which were stained under special conditions in different colors. For identification of free amino group containing phospholipids the ninhydrin reagent was used [12]. Finally, the chromatographic behavior and chemical properties of standard phospholipids were compared with those of experimental samples.

Gas–Liquid Chromatography of Fatty Acids

Fatty acid methyl esters of the total lipid extract of each semen sample obtained by the method of Bligh and Dyer [1] were prepared by reaction with 3 M acetyl chloride in methanol [12] in a boiling water bath for 1 h. Methyl esters were purified by one-dimensional, thin-layer chromatography in benzene. Methyl esters of fatty acids were quantitatively determined on a gas–liquid chromatograph "Chrom-5". The column of 3 × 2.0 (mm i.d. × m) containing 7.5% Silar-5CP (Serva) on Chromaton NAW-DMSC was used. For identification of fatty acids the standard fatty acid methyl esters (Sigma) were used. Temperature program was 140–250°C, at the rate of 2°C/min.

Protein was measured by the method of Lowry et al. [14]. The results were analyzed by the Student *t* test.

RESULTS

Phospholipid Composition of Sperm

The level of total phospholipid phosphorus of the spermatozoa significantly varied in healthy and infertile men. The great deviations of these values caused the lack of the statistically significant changes of the amount of total phospholipids in infertile men (data not presented). The amounts of individual phospholipids in sperm of normal men were in this order (Table 2): phosphatidyl choline (PC) > phosphatidyl ethanolamine (PE) > sphingomyelin (SM) >

Table 2. The individual phospholipid content in human spermatozoa (μg Pi/10^9 cells, mean ± SE)

Phospholipids	Healthy men (n = 5)	Infertile men (n = 11)
Phosphatidyl choline	22 ± 2.2	16.1 ± 3.1
Phosphatidyl ethanolamine	20 ± 2.7	7.0 ± 1.9*
Phosphatidyl serine	4.4 ± 0.7	8.2 ± 1.4*
Phosphatidyl inositol	2.2 ± 0.2	1.7 ± 0.5
Sphingomyelin	8.5 ± 1.5	12.7 ± 2.2
Lysophosphatidyl choline	1.8 ± 0.3	2.4 ± 0.5
Lysophosphatidyl ethanolamine	1.9 ± 0.5	2.0 ± 0.4
Lysophosphatidyl serine	1.3 (n = 1)	4.7 ± 1.2*
Diphosphatidyl glycerol	3.0 ± 0.6	2.7 ± 0.5
Nonidentified	3.2 ± 0.5	3.8 ± 0.8

*$p < .05$, compared to healthy men.

phosphatidyl serine (PS) > diphosphatidyl glycerol (DPG) > phosphatidyl inositol (PI) > lysophosphatidyl ethanolamine (LPE) > lysophosphatidyl choline (LPC). The significant level of lysophosphatidyl serine (LPS) was determined in spermatozoa of infertile men. The amount of PE decreased in sperm of infertile men more than twofold. In contrast, the level of PS in sperm of infertile men increased nearly twofold if compared to healthy subjects.

Phospholipid Composition of Seminal Plasma

There were no changes in the level of total phospholipid phosphorus and protein content in seminal plasma of infertile men compared to healthy subjects (data not presented), but the great variations of these values were noted. The relation of phospholipid amount to protein content in the seminal plasma of infertile men was not distinguished from that of the healthy subjects. The content of phospholipids in seminal plasma of fertile subjects can be ordered SM > PE > PC > PS > LPC > LPE > PI > LPS > DPG. The level of PS in the seminal plasma of infertile men was found to be increased more than twofold when compared to healthy subjects. Phosphatidic acid was found in trace amounts in seminal plasma of infertile patients only (Table 3).

Fatty Acid Composition of Ejaculates of Fertile and Infertile Men

In normal human semen, docosahexaenoic (22:6 n-3) and oleic acids (18:1 n-9) were the major unsaturated fatty acids. Palmitic acid (16:0) was the main saturated fatty acid; the quantity of stearic acid (18:0) took second place (Table 4). In infertile men the quantity of 18:0 was decreased more than twofold. The level of major n-3 polyunsaturated acid (PUFA) of human semen, 22:6 n-3, was decreased more than 3 times. The value of eicosapentaenoic acid (20:5 n-3) also significantly decreased more than 2 times. At the same time the amount of linolenic acid (18:3 n-6) and docosatetraenoic acid (22:4 n-6) increased 2 and 4 times, respectively. The amount of arachidonic acid (20:4 n-6) shows the trend to increase. The analysis of saturated/unsaturated fatty acids ratio shows that the total amount of saturated and unsaturated fatty acids was not affected. There was significant positive correlation between docosahexaenoic acid and sperm motility ($r = .82$, $p < .001$) and negative correlation between linolenic acid and sperm motility ($r = –.58$, $p < .05$).

Table 3. The individual phospholipids in human seminal plasma (μg Pi/10 mL of seminal plasma, mean ± SE)

Phospholipids	Healthy men (n = 5)	Infertile men (n = 11)
Phosphatidyl choline	17.1 ± 4.0	28.7 ± 5.2
Phosphatidyl ethanolamine	35.1 ± 7.6	28.3 ± 6.0
Phosphatidyl serine	13.4 ± 1.9	29.1 ± 6.9*
Phosphatidyl inositol	6.6 ± 0.5	5.6 ± 0.9
Sphingomyelin	52.1 ± 11.5	57.9 ± 9.8
Lysophosphatidyl choline	9.1 ± 1.7	12.8 ± 5.0
Lysophosphatidyl ethanolamine	8.8 ± 2.7	8.4 ± 1.8
Lysophosphatidyl serine	5.4 ± 1.4	10.2 ± 3.8
Diphosphatidyl glycerol	3.2 ± 1.3	4.9 ± 1.8
Phosphatidic acid	–	0.9 ± 0.1*
Nonidentified	12.4 ± 2.5	9.3 ± 2.6

*$p < .05$, compared to healthy men.

DISCUSSION

In infertile men some common changes were found in sperm: an increase in the level of PS and a significant decrease of PE. PE is the main nonlamellar phospholipid, which plays an important role in membrane fusion. Hence, the changes of PE in sperm of infertile men would be expected to have a definite impact on the alteration of sperm fertilizing ability.

LPS is not a characteristic constituent for many mammalian cells. Bruni [4] and other authors [5, 14] found this phospholipid in noticeable amounts in some pathological cases in granulocytes, mast cells, etc. and supposed that it served as a natural autacoid that promoted intercellular communications in coordinated reactions against perturbing stimuli. The reason for LPS appearance in infertile sperm and the functional role of this lipid here is not clear. This significant decrease of the PE content in the sperm of infertile men could be the result of (1) the peroxidative degradation of this phospholipid and (2) the inhibition of its synthesis from

Table 4. Composition of the main fatty acids in ejaculate of healthy and infertile men (mol% of total quantity, mean ± SE)

Fatty acid	Healthy men (n = 3)	Infertile men (n = 5)
Palmitic (16:0)	19 ± 1.58	26 ± 2.82*
Stearic (18:0)	17 ± 1.39	8.0 ± 1.16*
Oleic (18:1)	16 ± 0.39	16 ± 2.57
Linoleic (18:2)	3.76 ± 0.34	4.46 ± 1.02
Linolenic (18:3)	0.4 ± 0.07	1.0 ± 0.23*
Arachidonic (20:4 n-6)	1.14 ± 0.43	3.08 ± 1.19
Eicosopentaenoic (20:5 n-3)	2.31 ± 0.37	1.04 ± 0.20*
Docosahexaenoic (22:6 n-3)	16 ± 3.01	5.36 ± 0.47*
Lignoceric (24:0)	0.17 ± 0.03	0.34 ± 0.06*

*$p < .05$, compared to healthy men.

PS by decarboxylation rout. Reactive oxygen substances can damage the phospholipids by the free radical-induced oxidation of polyunsaturated fatty acids (PUFA) [8, 11, 20]. Thus, the loss of PE, a highly unsaturated phospholipid, in sperm of infertile men could be due at least partly to its peroxidative decomposition. This idea is supported by the drastic fall of the major n-3 PUFA, which is an important constituent of PE and other phospholipids—docosahexaenoic (22:6 *n*-3) and eicosopentaenoic (20:5 *n*-3) acids. The last fact could be easily explained by the free radical mechanism of PUFA destruction. PS is one of the main precursors of PE (for review see [21]). Probably an inhibition of the PS–decarboxylase pathway [3] could cause the high level of PS and decreased level of PE in infertile sperm, further investigations are necessary to clarify this question. The nature of linolenic acid (18:3 *n*-6) enhancement in infertile semen is unknown. Sperm infertility is associated with the drastic loss of phosphatidyl ethanolamine and *n*-3 PUFA with simultaneous enhancement of phosphatidyl serine and some *n*-6 PUFA.

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