Chapter 5

Phospholipid Composition of Human Sperm and Seminal Plasma in Relation to Sperm Fertility

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Abstract

Some infertile men have idiopathic infertility with near normal spermogram and have no obvious propagations of any pathological processes. The sperm lipid composition of infertile men with normozoospermia is poorly understood. Many investigators have shown that even subnormal motile densities and morphology did not predict the infertile male. We suggested that decreased sperm fertile potential can be associated with alteration of sperm lipid composition and content. To elucidate this problem we investigated the phospholipid and fatty acid composition of human normozoospermic semen in relation to its fertility. We found an approximately threefold decrease of phosphatidylethanolamine (PE) in spermatozoa of infertile normozoospermic men. In contrast the level of phosphatidylserine (PS) and simultaneously of lysophosphatidylserine (LPS) increased nearly twofold compared to healthy subjects. As a result, the phospholipid amounts in the spermatozoa of infertile men were significantly different compared to healthy men. The level of PS in seminal plasma of infertile men was found to increase more than twofold. The phosphatidic acid was found in small amounts in seminal plasma of infertile patients only. The phospholipid percentage in the seminal plasma of infertile men was significantly different compared to healthy men. In infertile men the quantity of 18:0 decreased more than twofold. The level of docosahexaenoic acid decreased more than threefold. The value of 20:5n-3 also decreased more than twofold. The amount of 18:3n-6 doubled. The amount of 20:4n-6 showed the trend to increase. The ratio of total amount of saturated to unsaturated fatty acids was not affected. There was a significant positive correlation between 22:6n-3 content and sperm motility and a negative correlation between 18:3n-6 amount and sperm motility.

Introduction

It is known that more than 15% of reproductive age couples present with infertility (1). A number of pathological states were identified as causes of male infertility including gene mutations, aneuploidy, infectious diseases, ejaculatory duct occlusion,

varicocele, radiation, chemotherapy, and erectile dysfunction (2). Nearly half of infertile men have infertility with unknown etiology identified as idiopathic. Lipids are thought to play a major role in regulating spermatozoa fertility.

Human semen is formed from the secretions of different glands, and usually two main fractions can be obtained by semen centrifugation: spermatozoa and seminal plasma. Most semen protein (about 85%) is found in soluble fraction and nearly 7% in spermatozoa. On the contrary, phospholipids are largely bound to the fraction of spermatozoa (nearly 45% of total lipid phosphorus). This fraction is poor in cholesterol and has a cholesterol-to-phospholipid molar ratio of about 0.2 (3). It was shown that cholesterol was the predominant sterol both in spermatozoa (107 +/- 7 nmol/10^8 spermatozoa) and in seminal plasma (0.83 +/- 0.1 μmol/ml) (4). As shown by many authors, all the semen fractions have specific lipid content. We studied phospholipid composition in spermatozoa of the individual semen of healthy men, which showed 32.9% phosphatidylcholine (PC), 26.6% phosphatidylethanolamine (PE), 11.7% sphingomyelin (SM), 7.3% phosphatidylserine (PS), 6.7% diphosphatidylglycerol (DPG), 3.8% phosphatidylinositol (PI), 3% lysophosphatidylcholine (lysoPC), and 2.9% lysophosphatidylethanolamine (lysoPE).

The phospholipid content of seminal plasma differed markedly from spermatozoa. It contained 32.9% SM, 19.8% PE, 11.1% PC, 8.3% PS, 5.4% lysoPE, 5.2% lysoPC, 3.6% lysophosphatidylserine (lysoPS), and 1.7% DPG (5,6).

It is well established that different lipids play a significant role in sperm functional activity. Semen analysis of mammals has shown that processing, capacitation, and acrosome reaction are associated with multiple specific modifications of phospholipid composition of spermatozoa plasma membrane (7–9).

PI is found to play a role in acrosomal reaction of human spermatozoa. After induction, PI is hydrolyzed by phospholipase C with formation of diacylglycerol and inositolphosphates (10). Progesterone is known to induce acrosomal reaction, whereas cholesterol is a major inhibitor of this reaction.

Cholesterol inhibited the progesterone-induced acrosome reaction when it was added *in vitro* to sperm during capacitation and when it was added with progesterone during the induction of acrosome reaction. Similarly acrosome reaction that was induced by db-cAMP was also inhibited by cholesterol in concentration of 0.2 μg/mL. Cholesterol's inhibition of induced acrosome reaction was independent of progesterone concentration. Cholesterol inhibited acrosome reaction in a noncompetitive manner by modifying the structure of the sperm plasma membrane, which prevented exposure of the progesterone surface receptor to progesterone binding (11).

Capacitation is also a lipid dependent process, namely, a complex series of molecular events that occurs in sperm after epididymal maturation and confers on spermatozoa the ability to fertilize an egg. Capacitation correlates with cholesterol efflux from the sperm plasma membrane, increased membrane fluidity, modulations in intracellular ion concentrations, hyperpolarization of the sperm plasma membrane, and increased protein tyrosine phosphorylation. These molecular events are required for the subsequent induction of hyperactivation and the acrosome reaction (12).

The lipid composition of the sperm membrane has a significant effect upon the functional characteristics of spermatozoa. Changes of sperm lipid composition cause alterations of sperm functional characteristics. Damage induced by reactive oxygen species generated by spermatozoa endogenously has been proposed as a major factor in male infertility (13). It is known that polyunsaturated fatty acids (PUFA) are the main substrate of lipid peroxidation. Recently, we and others have shown that docosahexaenoic acid (DHA, 22:6n-3) is a major PUFA in human spermatozoa (14,15).

The essential fatty acid DHA is a minor component of the Western diet but a major fatty acid in human testis and semen. In mature spermatozoa, the physical and fusogenic properties of the plasma membrane are probably influenced by its particular fatty acid composition. According to some authors, DHA accounts for up to 30% of fatty acids esterified in phospholipids and 73% of all PUFA (14,16). DHA is thought to play a major role in regulating membrane fluidity in spermatozoa. It serves as a main substrate of lipid peroxidation in human spermatozoa. It was shown that spermatozoa motility depends on DHA (17). We also published the results of an investigation that showed the existence of positive correlation between sperm motility and DHA amount in spermatozoa (14).

Changes in the proportions of the various lipid components in spermatozoa were investigated throughout the development and reproductive period of many male beings, including men. Sperm motility and *in vivo* fertility are known to depend on sperm lipid composition. This question was studied by many investigators and it was assumed that seminal lipid composition played a major role in providing normal realization of different processes of sperm fertile function.

It was shown that total lipid concentration was elevated in the seminal plasma of oligo- and azoospermic men. The total cholesterol content was comparatively higher in the seminal plasma of azoospermic men than in that of normo- and oligospermic men. In general, infertility was associated with increased seminal concentrations for most of the neutral lipid classes. However, total phospholipids and most of the phospholipid classes were diminished in the seminal plasma of oligo- and azoospermic men and in the spermatozoa of oligospermic men. Authors suggest that there is a positive correlation between seminal phospholipids and fertility and a negative correlation between seminal neutral lipids and fertility (18).

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 (19). Many investigators have shown that even subnormal motile densities and morphology did not predict the infertile male (20). It is known that some infertile men have idiopathic infertility with near normal spermogram and have no obvious propagations of any pathological processes. We suggested that decreased sperm fertile potential can be associated with alteration of sperm lipid composition and content. To elucidate this question we investigated the phospholipid and fatty acid composition of human normozoospermic semen in relation to its fertility.

Material 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.

Sperm Count and Separation

Sperm were counted immediately after liquefaction of semen according to the WHO protocol (21) with some modifications. For evaluation of the number of sperm, two 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 shows the number of motile sperm. Dead sperm and abnormal cells were determined after staining by eosin and nigrosine. 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 suspension, which contained 10–50 $\times$ 10^6 sperm/mL. This suspension was then 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 vol/vol) according to Bligh and Dyer (22). 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. 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 (23). The lipid extract was stored in a small volume of chloroform at –20°C.

Phospholipids were analyzed by two-dimensional, high-resolution, micro thin-layer chromatography 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 (70:30:10:5:4:1, by vol) (24) as solvent systems. For phospholipid development we used 60 $\times$ 60-mm^2 plates. To determine the quantity of the phospholipid phosphorus, the molybdate spray reagent was used (25). This method permitted us to analyze the phospholipid composition of individual samples of semen.

Individual phospholipids were revealed with molybdate (25) and malachite green reagents (26). The anthrone reagent (27) was also used to determine phospholipid, sulpholipid, and glycolipid containing spots, which were stained under special condi-

tions in different colors. For identification of free amino groups containing phospholipids, the ninhydrin reagent was used (28). 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 (22) were prepared by reaction with 3 M HCl in methanol (29) in a boiling water bath for 1 h. Methyl esters were purified by one-dimensional, high-resolution, micro thin-layer chromatography in benzene. Methyl esters of fatty acids were quantitatively determined on a gas–liquid chromatograph (Chrom-5, Czech). The column of 2 m x 3 mm (L x i.d) 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.* (30). The results were analyzed by the Student *t*-test.

Results

Two groups of men with very similar semen morphologic characteristics were investigated, healthy men ($n = 8$) and infertile men ($n = 16$). In both groups semen volume was 4.0 (SD 0.4) mL *versus* 4.2 (SD 0.4) mL; sperm count was 103 (SD 15) $\times 10^6$/mL *versus* 103 (SD 9) $\times 10^6$/mL; and total sperm count in whole ejaculate was 413 (SD 67) $\times 10^6$ *versus* 415 (SD 47) $\times 10^6$. The amount of motile sperm cells, 68 (SD 9) $\times 10^6$ *versus* 52 (SD 7) $\times 10^6$, and quantity of dead cells, 31 (SD 7) $\times 10^6$ *versus* 42 (SD 6) $\times 10^6$, were statistically the same. Only the quantity of abnormal cells was significantly higher in infertile men: 26 (SD 6) $\times 10^6$/mL *versus* 43 (SD 5) $\times 10^6$/mL ($P < 0.05$).

The level of total phospholipid phosphorus 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 the following order (Table 5.1): PC > PE > SM > PS > DPG > PI > lysoPE > lysoPC > lysoPS. We found a significant (about threefold) decrease in the PE amount in spermatozoa of infertile men. In contrast the level of PS and simultaneously of lysoPS increased nearly twofold compared to healthy subjects ($P < 0.05$). As a result the order of the phospholipid amounts in the spermatozoa of infertile men became as follows: PC > SM > PS > PE > lysoPS > DPG > lysoPC > lysoPE > PI.

We did not find changes in the level of total protein and phospholipid phosphorus and amount in seminal plasma of infertile men compared to healthy subjects (Table 5.2). However, we noted great individual deviations of these values. The main phospholipid of seminal plasma was SM. The content of individual phospholipids in seminal plasma significantly differed from that of spermatozoa and was ordered as fol-

Table 5.1
The Individual Phospholipid Content of Human Spermatozoa (μg Pi/10^9 cells, *mean ± SE*)

Phospholipids	Healthy men (n = 5)	Infertile men (n = 11)
Phosphatidylcholine	22.0 ± 2.2	16.1 ± 3.1
Phosphatidylethanolamine	20.0 ± 2.7	7.0 ± 1.9*
Sphingomyelin	8.5 ± 1.5	12.7 ± 2.2
Phosphatidylserine	4.4 ± 0.7	8.2 ± 1.4*
Diphosphatidylglycerol	3.0 ± 0.6	2.7 ± 0.5
Phosphadylinositol	2.2 ± 0.2	1.7 ± 0.5
Lysophosphatidylcholine	1.8 ± 0.3	2.4 ± 0.5
Lysophosphatidylethanolamine	1.9 ± 0.5	2.0 ± 0.4
Lysophosphatidylserine	1.3 (n = 1)	4.7 ± 1.2*

*$p < 0.05$, compared to healthy men.

Table 5.2
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)
Sphingomyelin	52.1 ± 11.5	57.9 ± 9.8
Phosphatidylethanolamine	35.1 ± 7.6	28.3 ± 6.0
Phosphatidylcholine	17.1 ± 4.0	28.7 ± 5.2
Phosphatidylserine	13.4 ± 1.9	29.1 ± 6.9*
Lysophosphatidylcholine	9.1 ± 1.7	12.8 ± 5.0
Lysophosphatidylethanolamine	8.8 ± 2.7	8.4 ± 1.8
Phosphadylinositol	6.6 ± 0.5	5.6 ± 0.9
Lysophosphatidylserine	5.4 ± 1.4	10.2 ± 3.8
Diphosphatidylglycerol	3.2 ± 1.3	4.9 ± 1.8
Phosphatidic acid	—	0.9 ± 0.1*

*$p < 0.05$, compared to healthy men.

lows: SM > PE > PC > PS > lysoPC > lysoPE > PI > lysoPS > DPG. The level of PS in seminal plasma of infertile men was found to increase more than twofold when compared to healthy men. It is interesting to note that phosphatidic acid was found in trace amounts in seminal plasma of infertile patients only.

The major unsaturated fatty acids in normal human semen were docosahexaenoic (22:6n-3) and oleic (18:1n-9) acids (Table 5.3). Palmitic acid (16:0) was the main saturated fatty acid; stearic acid (18:0) took second place. In infertile men the quantity of 18:0 was decreased more than twofold. The level of major n-3 PUFA of human semen, 22:6n-3, was decreased more than three times. The value of eicosapentaenoic acid (20:5n-3) also significantly decreased more than two times. At the same time the amount of γ-linolenic acid (18:3n-6) increased two times. The amount of arachidonic acid (20:4n-6) showed the trend to increase. The analysis of saturated/unsaturated

Table 5.3
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 (c16:0)	19.0 ± 1.58	26.0 ± 2.82
Stearic (c18:0)	17.0 ± 1.39	8.0 ± 1.16*
Oleic (c18:1n-9)	16.0 ± 0.39	16.0 ± 2.57
Linoleic (c18:2n-6)	3.76 ± 0.34	4.46 ± 1.02
Linolenic (c18:3n-3)	0.4 ± 0.07	1.0 ± 0.23*
Arachidonic (c20:4n-6)	1.14 ± 0.43	3.08 ± 1.19
Eicosopentaenoic (c20:5n-3)	2.31 ± 0.37	1.04 ± 0.20*
Docosahexaenoic (c22:6n-3)	16.0 ± 3.01	5.36 ± 0.47*
Lignoceric (c24:0)	0.17 ± 0.03	0.34 ± 0.06*

*$p < 0.05$, compared to healthy men.

fatty acids ratio shows that the total amount of saturated and unsaturated fatty acids was not affected. There was a significant positive correlation between 22:6n-3 and sperm motility ($r = 0.82$, $P < 0.001$) and a negative correlation between γ-linolenic acid and sperm motility ($r = -0.58$, $P < 0.05$).

Discussion

In infertile men some common changes were found in spermatozoa and seminal plasma: 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.

LysoPS is not a characteristic constituent for many mammalian cells. Bruni and other authors (31,32) 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 lysoPS appearance in infertile sperm and the functional role of this lipid here is not clear. The significant decrease of the PE content in the sperm of infertile men could be the result of (i) the peroxidative degradation of this phospholipid and (ii) the inhibition of its synthesis from PS by decarboxylation. Reactive oxygen substances can damage the phospholipids by the free radical-induced oxidation of PUFA (33–35). 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 (22:6n-3 and 20:5n-3), which are important constituents of PE and other phospholipids. 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 (36)). Probably an inhibition of the PS-decarboxylase

pathway (37) 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 γ-linolenic acid (18:3n-6) enhancement in infertile semen is unknown. The results of these investigations show that sperm infertility is associated with the drastic loss of PE and n-3 PUFA with simultaneous enhancement of PS and some n-6 PUFA.

It is interesting to note that there were some attempts to correct male infertility by dietary 22:6n-3 (38,39, Chapter 4 in this book). The effects of supplementation with DHA on DHA levels in serum, seminal plasma, and sperm of asthenozoospermic men as well as on sperm motility were examined in a randomized, double-blind, placebo-controlled manner (38,39). Asthenozoospermic men (n = 28; less or = 50% motility) were supplemented with 0, 400, or 800 mg DHA per day for three months. Sperm motility and the fatty acid composition of serum, seminal plasma, and sperm phospholipid were determined before and after supplementation. There was no effect of DHA supplementation on sperm motility. The results showed that dietary supplementation resulted in increased serum and possibly seminal plasma phospholipid DHA levels without affecting the incorporation of DHA into the spermatozoa phospholipid in asthenozoospermic men. This inability of DHA to be incorporated into sperm phospholipid was most likely responsible for the observed lack of DHA supplementation effect on sperm motility.

This fact can be partly explained by the results recently published by Retterstol *et al.* (40, Chapter 2 in this book). They studied the synthesis of 22:6n-3 and 22:5n-6 in isolated human testicular cells. [1-^{14}C]20:4n-6, [1-^{14}C]20:5n-3, [1-^{14}C]22:4n-6, and [1-^{14}C]22:5n-3 were incubated in a "crude" cell suspension (consisting of a mixture of the cells in the seminiferous tubule) and in fractionated pachytene spermatocytes and round spermatids. The esterification of fatty acids in lipid and phospholipid classes and the fatty acid chain elongation and desaturation were measured. The "crude" cell suspension metabolized the fatty acids more actively than did the fractionated germ cell suspension, indicating that types of cell other than the germ cells are important for fatty acid elongation and desaturation and thus the production of [^{14}C]22:6n-3. This finding is in agreement with previous results in rats that indicated that the Sertoli cells are the most important type of cell for the metabolism of essential fatty acids in the testis. Some [1-^{14}C]20:5n-3 was elongated to [^{14}C]22:5n-3 in the fractionated germ cells, but very little was elongated further to [^{14}C]24:5n-3, possibly restricting the formation of [^{14}C]22:6n-3. In the fractionated germ cells, the fatty acid substrates were recovered primarily in the phospholipid fraction, indicating an incorporation in the membranes, whereas in the "crude" cells, more substrates were esterified in the triacylglycerol fraction. In the phospholipids, more radioactivity was recovered in PC than in PE and more radioactivity was recovered in PE than in PI or PS. These results show that many types of cells play a role in the fatty acid and phospholipid synthesis, and in many cases the deep disturbances of different lipids and particularly PUFA synthesis and metabolism cause the male infertility.

Summary

About threefold decrease of PE amount in spermatozoa of infertile normozoospermic men was found when compared to healthy men. In contrast the level of PS and simultaneously of lysoPS increased nearly twofold when compared to healthy subjects. As a result the order of the phospholipid amounts in the spermatozoa of infertile men changed significantly .

The level of PS in seminal plasma of infertile men was found to increase more than twofold. The phosphatidic acid was found in small amounts in seminal plasma of infertile patients only. The order of the phospholipid quantities in seminal plasma of infertile men significantly changed compared to healthy men.

In infertile men the quantity of 18:0 decreased more than twofold. The level of DHA decreased more than three times. The value of 20:5n-3 also significantly decreased, more than twofold. The amount of 18:3n-6 increased two times. The amount of 20:4n-6 showed the trend to increase. The ratio of total amount of saturated to unsaturated fatty acids was not affected. The significant positive correlation between 22:6n-3 content and sperm motility and negative correlation between 18:3n-6 amount and sperm motility were shown. The results of this investigation suggest that decreased sperm fertile capacity can be associated with alteration of sperm lipid composition and content.

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