Pattern of neutral and phospholipids in the semen of normospermic, oligospermic and azospermic men

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Summary. Total lipid concentration was elevated in the seminal plasma of oligo- and azospermic men. The total cholesterol content was comparatively more in the seminal plasma of azospermic 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 azospermic men and in the spermatozoa of oligospermic men. We suggest that there is a positive correlation between seminal phospholipids and fertility and a negative correlation between seminal neutral lipids and fertility.

Introduction

Lipids play an important role in the spermatozoa and thus on male fertility. Neutral lipids, particularly triacylglycerols and diacylglycerols, serve predominantly as the source of oxidizable energy (White *et al.*, 1976; Evans & Setchell, 1979). It has been suggested that cholesterol modifies the lipid bilayer of the sperm plasma membrane and prevents its fusion with the outer acrosomal membrane (Davis, 1978). Phospholipids form an integral part of the sperm membrane (Poulos *et al.*, 1973) and may be involved in membrane transport and enzyme activities (White *et al.*, 1976). Phospholipids also have an important role in meeting the energy requirements during cell proliferation and maturation of spermatozoa (see Mann, 1964). Davis & Byrne (1980a) suggest that the cholesterol:phospholipid ratio plays an important role in the fertilizing ability of spermatozoa. It may therefore be expected that infertility might be associated with altered lipid metabolism in the semen. However, little work is available on the pattern of different classes of lipids and their fractions in the seminal plasma and spermatozoa of infertile men. We have therefore studied these patterns in the semen of normospermic, oligospermic and azospermic men.

Materials and Methods

Semen samples were collected from normal, healthy human subjects with proven fertility as well as from men proven to be oligospermic and azospermic. The men were 25–35 years of age and there were 10 men in each group. Spermatozoa were counted as directed in the WHO protocol (Belsey *et al.*, 1980). Men with a count of 40×10^6 spermatozoa/ml and 60–70% motility were considered as normal. Men with sperm count of $< 20 \times 10^6$ /ml and 30–40% motility were considered as oligospermic. Men with no spermatozoa in their semen were classified as azospermic.

Spermatozoa and seminal plasma were separated by centrifuging the semen at 20 000 g at 4°C for 30 min. The supernatant was considered as seminal plasma and the sediment as spermatozoa. Lipids from both fractions were extracted in chloroform:methanol (2:1, v/v) by the method of Folch *et al.* (1957): 0.01% butylated hydroxytoluene (BHT) was added to the extraction mixture as an antioxidant.

The lipid extract was concentrated *in vacuo* at 40–45°C, resuspended in chloroform:methanol (2:1, v/v) containing 4% water and evaporated at 40–45°C *in vacuo*. This process was repeated 3 times and residues were suspended in 100 ml chloroform:methanol (2:1, v/v) containing 0.01% BHT. This lipid extract was used for the analysis of total lipids, neutral lipids and phospholipids.

Total lipid was quantified by the gravimetric method. Cholesterol was estimated by the method of Hanel & Dam (1955). Total glyceride glycerol was determined by the procedure described by Van Handel & Zilversmit (1957). Total phospholipid phosphorus was estimated by the method of Fiske & Subbarow (1925) as described by Marinetti (1962) and the values so obtained were multiplied by 25 to obtain phospholipid values (Bieri & Prival, 1965).

Neutral lipid fractions were resolved by thin-layer chromatography on 500 µm plates of silica gel G. Lipid extracts containing 50–60 µg glyceride glycerols were well separated. The plates were developed in the solvent system proposed by Mangold (1966). To begin with, the plates were developed to a height of 7 cm in the first solvent system of *n*-hexane:diethyl ether:glacial acetic acid (60:40:1, by vol.), air-dried and developed in the second solvent system of *n*-hexane:diethyl ether:glacial acetic acid (90:100:1, by vol.) to a height of 15 cm. After air drying, various fractions were identified in an iodine chamber by comparing with authentic standards (Sigma Chemical Company, St Louis, MO, U.S.A.). For good separation of monoacylglycerol, a line was drawn below the diacylglycerol fraction and the plate was again run upto this line in the third solvent system of *n*-hexane:diethyl ether:glacial acetic acid (30:70:1, by vol.). Glyceride glycerol spots were scraped and eluted with chloroform 3 times and estimated by the method of Van Handel & Zilversmit (1957). The recovery of different classes of glyceride glycerols was more than 95%.

Phospholipids were also fractioned by one-dimensional thin-layer chromatography using the modified solvent system of Abramson & Blecher (1964), consisting of chloroform:methanol:7 N-ammonia (75:25:5, by vol.). To avoid degradation of plasmalogens, all the chromatographic operations were carried out at 4°C (Poulous *et al.*, 1973). Lipid extract containing 10–15 µg phosphorus was well separated into the various fractions. The recovery of various fractions of phospholipids was more than 90%. Spots were identified in iodine chamber by comparison with R_f values of known standards (Calbiochem, Lucerne, Switzerland). The fractions were eluted in a solvent mixture of chloroform:methanol:formic acid:water (97:97:4:2, by vol.). The inorganic phosphorus present in each fraction was estimated by the method described above, after digestion with 70% perchloric acid for 15 min.

The data were statistically analysed using Student's *t* test.

Results

As shown in Table 1, total lipid was higher in the seminal plasma and spermatozoa of oligospermic subjects and seminal plasma of azoospermic subjects, than in normal men.

The results for glyceride glycerols are presented in Table 2. There was little difference in the concentrations of mono-, di- and triacylglycerols in seminal plasma and spermatozoa of normal subjects. Azoospermia was associated with an increased concentration of monoacylglycerol, but seminal plasma of oligospermic men had lower concentrations. Nevertheless, the spermatozoa of oligospermic men showed increased concentration of monoacylglycerol. Diacylglycerol was markedly elevated in the seminal plasma of oligo- and azoospermic men and in the spermatozoa of oligospermic men. Triacylglycerol content was significantly increased in azoospermia. While seminal plasma triacylglycerol did not show any change in oligospermia, there was an appreciable increase in the spermatozoa.

Table 1. Total lipid and various classes of lipids in the seminal plasma and spermatozoa of normal and infertile men

Samples	Total lipids	Total cholesterol	Total glycerides	Total phospholipids
Seminal plasma (mg/100 ml)				
Normal	275.10 ± 9.46	92.88 ± 2.38	81.95 ± 2.07	73.48 ± 4.20
Oligospermic	360.54 ± 12.40***	94.36 ± 3.97	117.67 ± 4.61***	61.61 ± 2.57*
Azoospermic	509.29 ± 17.52***	152.25 ± 6.50***	270.41 ± 9.30***	54.40 ± 2.13***
Spermatozoa (mg/g wet wt)				
Normal	57.90 ± 2.63	17.76 ± 0.75	12.89 ± 0.45	17.10 ± 0.81
Oligospermic	69.02 ± 3.13*	17.91 ± 0.90	31.07 ± 1.41***	12.83 ± 0.53***

Each value is the mean ± s.e.m. of 10 estimations.

P* < 0.05; **P* < 0.001, compared with values for normal men.

Table 2. Concentrations of mono-, di- and triacylglycerols in the semen of normal and infertile men

Samples	Monoacylglycerols	Diacylglycerols	Triacylglycerols
Seminal plasma (mg/100 ml)			
Normal	31.49 $\pm$ 0.88	28.22 $\pm$ 0.71	22.08 $\pm$ 1.77
Oligospermic	26.43 $\pm$ 1.03***	72.35 $\pm$ 2.83***	18.42 $\pm$ 0.72
Azoospermic	147.86 $\pm$ 5.09***	72.50 $\pm$ 3.57***	43.23 $\pm$ 1.48***
Spermatozoa (mg/g wet wt)			
Normal	3.70 $\pm$ 0.13	4.80 $\pm$ 0.17	4.33 $\pm$ 0.15
Oligospermic	5.32 $\pm$ 0.26***	18.04 $\pm$ 0.82***	7.63 $\pm$ 0.34***

Each value is the mean $\pm$ s.e.m. of 10 estimations.

****P* < 0.001, compared with values for normal men.

As shown in Table 3, in normal men, sphingomyelin was the major phospholipid fraction in the seminal plasma, followed by phosphatidylserine and phosphatidylethanolamine. Phosphatidylcholine was predominant in the spermatozoa followed by phosphatidylethanolamine and sphingomyelin. There was no change in this pattern in the infertile men, but sphingomyelin was markedly decreased in the seminal plasma of oligo- and azoospermic men and in the spermatozoa of oligospermic men. Similarly, ethanolamine plasmalogen, phosphatidylinositol and phosphatidylserine were decreased in the seminal plasma of oligo- and azoospermic subjects. In oligospermic subjects the spermatozoa showed decreased concentrations of phosphatidylcholine and -ethanolamine and sphingomyelin.

Discussion

The results obtained in the present study suggest accumulation of lipids in the semen of oligo- and azoospermic men. The increased concentration of lipids in the semen of infertile men is mainly due to the accumulation of neutral lipids as phospholipids were decreased. This may be due to poor or non-utilization of glyceride glycerols by the spermatozoa of such men. Turner & Johnson (1971) reported that lipids accumulate when the spermatozoa fail to utilize glyceride glycerols. Havel (1972) also reported that complete absence of spermatozoa (azoospermia) results in non-utilization of glyceride glycerols and their accumulation in the seminal plasma. Minassian & Terner (1966) reported a high turnover of diacyl- and triacylglycerols in human spermatozoa, and these lipids serve as a source of endogenous energy for the spermatozoa (White *et al.*, 1976). In the present study, these fractions were high in the spermatozoa of normal men, and the concentration of glyceride glycerols is inversely proportional to the sperm count. On the other hand the phospholipid concentration showed a direct correlation with sperm number. Havel (1970) also reported this type of relation between sperm concentration and phospholipids.

The concentration of diacylglycerol was very high in the spermatozoa and seminal plasma of oligospermic subjects in the present study. The seminal plasma of azoospermic subjects also exhibited high concentration of diacylglycerols but the monoacylglycerol in these subjects was 100% more than that of diacylglycerols. Diacylglycerol is utilized for the synthesis of phospholipids and triacylglycerols (Sun & Horrocks, 1971; White *et al.*, 1973). The accumulation of diacylglycerol with a simultaneous decrease in phospholipids in infertile men may indicate a decreased rate of conversion of diacylglycerol to phospholipids in these subjects, but further studies are required.

Table 3. The distribution pattern of phospholipids in the seminal plasma and spermatozoa of normal and infertile men

Samples	Phosphatidyl- choline	Phosphatidyl- ethanolamine	Sphingomyelin	Ethanolamine plasmalogen	Phosphatidyl- serine	Choline plasmalogen	Phosphatidyl- inositol	Miscellaneous lipids
Seminal plasma (mg/100 ml)								
Normal	10.30 ± 1.67	6.70 ± 3.16	31.10 ± 0.20	8.18 ± 0.20	7.19 ± 0.22	0.66 ± 0.12	0.73 ± 0.13	8.08 ± 0.20
Oligospermic	8.73 ± 0.19	4.90 ± 0.20	26.26 ± 0.20***	7.02 ± 0.20***	6.18 ± 0.20***	0.55 ± 0.13	0.62 ± 0.09*	6.77 ± 0.27*
Azoospermic	7.79 ± 0.22	4.41 ± 0.24	23.29 ± 0.20***	6.10 ± 0.20***	5.44 ± 0.20***	0.48 ± 0.07	0.54 ± 0.10***	5.88 ± 0.19***
Spermatozoa (mg/g wet wt)								
Normal	6.07 ± 0.21	3.53 ± 0.20	3.18 ± 0.20	1.56 ± 0.27	0.72 ± 0.13	0.48 ± 0.09	0.21 ± 0.03	1.09 ± 0.35
Oligospermic	4.63 ± 0.20***	2.72 ± 0.19*	2.26 ± 0.19**	1.17 ± 0.22	0.54 ± 0.09	0.36 ± 0.06	0.15 ± 0.06	0.82 ± 0.16

Each value is the mean ± s.e.m. of 10 estimations.
P* < 0.05; *P* < 0.01; ****P* < 0.001 compared with value for normal men.

The amounts of phospholipids reported in the present study on seminal plasma and spermatozoa of normal subjects are equivalent to those of earlier reports (Poulos & White, 1973; Jones *et al.*, 1979; Umapathy *et al.*, 1980). While sphingomyelin is the predominant phospholipid in the seminal plasma, phosphatidylcholine is the major fraction in the spermatozoa; this trend was also present in oligo- and azoospermic men. The decrease in phospholipids in the seminal plasma of oligo- and azoospermic subjects was mainly due to the reduction in sphingomyelin, ethanolamine plasmalogen, phosphatidylserine and phosphatidylinositol. Similarly, the reduction of total phospholipids in the spermatozoa of oligospermic subjects was mainly due to the decrease in phosphatidylcholine, phosphatidylethanolamine and sphingomyelin.

Since the phospholipids are integral membrane components, they may be involved in membrane permeability and enzyme reactions (White *et al.*, 1976). Vanitha Kumari & Govindarajulu (1981) reported decreased activities of transaminases and phosphomonoesterases in the seminal plasma of oligo- and azoospermic men. A decrease in phospholipid concentration in the spermatozoa of oligospermic men will result in an altered ratio of cholesterol:phospholipids, despite an unaltered cholesterol level. This will result in altered membrane cohesiveness and thus a decreased fertilizing capacity (Davis & Byrne, 1980a). The accumulation of cholesterol in the seminal plasma of azoospermics may be due to non-utilization of available cholesterol to integrate with the sperm membrane (Oldfield & Chapman, 1972; Phillips & Finer, 1974; Darin-Bennett & White, 1976).

Normal concentrations of phosphatidylserine and -choline in the spermatozoa play a vital role in the fertilizing ability of the spermatozoa (Papahadjopoulos *et al.*, 1973, 1974; Davis & Byrne, 1980b). Hence, the decrease in the concentrations of phosphatidylcholine in the spermatozoa of the oligospermic men in the present study would be expected to have a definite impact on the fertilizing ability of these spermatozoa.

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