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The fatty acid composition of phospholipids of spermatozoa from infertile patients

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The lipid composition of the sperm membrane has a significant effect upon the functional characteristics of spermatozoa. In the present study we investigated the fatty acid (FA) composition of subpopulations of spermatozoa separated on a discontinuous Percoll gradient (47:90%) and the FA composition of phospholipids (PL) of sperm heads and tails in both normal and abnormal semen samples. In normozoospermic samples, polyunsaturated fatty acids (PUFA) represented 34.0 ± 1.3 (mean ± SE, mole %) and 25.6 ± 1.2% of total FA of PL of the 47 and 90% Percoll fractions respectively. Docosahexaenoic acid (22:6ω3, DHA) contributed to more than 60% of total PUFA. DHA was significantly lower in both the 47% ($P < 0.05$) and the 90% ($P < 0.01$) Percoll fractions of oligozoospermic samples and in the 90% Percoll layer of asthenozoospermic samples ($P < 0.01$), compared with normozoospermic samples. The ω6/ω3 ratio was significantly increased in both Percoll fractions of samples with oligozoospermia (47%, $P < 0.001$ and 90%, $P < 0.001$) or with asthenozoospermia (47%, $P < 0.05$ and 90%, $P < 0.001$) compared with normozoospermic samples. The oxidative potential index (OPI) of spermatozoa recovered from the 47% Percoll layer was significantly higher ($P < 0.0001$) than of those recovered from the 90% Percoll. Mean melting point (MMP), an index of membrane fluidity, was significantly lower in head than in tails ($P < 0.01$) of spermatozoa, and also in both the 47% ($P < 0.01$) and 90% ($P < 0.001$) Percoll fractions of normozoospermic samples in comparison with oligozoospermic samples. The MMP was significantly higher ($P < 0.05$) in samples of patients with idiopathic oligo/asthenozoospermia, varicocele, and male accessory gland infection (MAGI). These differences in FA composition of PL in subpopulations of human spermatozoa, and in their heads and tails may be related to sperm maturity and to differences in physiological function.

Key words: DHA/fatty acids/male infertility/spermatozoa

Introduction

The membrane structure of spermatozoa plays a pivotal role for successful fertilization, since both the acrosome reaction and sperm–oocyte fusion are membrane-associated events (Yanagimachi, 1981; Clegg, 1983). The lipids of the spermatozoa have been suggested to be important for the viability, maturity, and function of spermatozoa (Brian, 1981; Sebastian *et al.*, 1987). Indeed, Hall *et al.* (1991) have found that membrane fluidity and the phospholipid (PL) composition of the membrane may change during epididymal maturation. The spermatozoa may be susceptible to peroxidative damage by virtue of their high content of unsaturated fatty acids and the relative paucity of cytoplasmic enzymes for scavenging the reactive oxygen species that initiate lipid peroxidation (Alvarez *et al.*, 1987; Alvarez and Storey, 1989; Aitken *et al.*, 1991, 1992; Zalata *et al.*, 1995a,b). A major consequence of lipid peroxidation is a change in membrane fluidity which, in turn, inhibits events during gamete fusion (Aitken, 1989). In addition, changes in lipid structure may result in differences in the expression of membrane receptors, e.g. C-met receptors (Depuydt *et al.*, 1996).

In view of the importance of lipid structure, several studies have described the lipid composition of spermatozoa from different species. In monkeys, docosahexaenoic acid (22:6ω3, DHA) was found to be highly concentrated in spermatozoa, and oleic and palmitic acid were the predominant monounsaturated and saturated fatty acids (FA) respectively (Lin *et al.*, 1993). In contrast, Ahluwalia and Holman (1969) found only trace amount of DHA in human spermatozoa. The majority of available information on the FA composition of spermatozoa was obtained from pooled samples, and the differences in FA of PL fractions of different populations of spermatozoa of normal and abnormal samples has not been determined previously.

The aim of the study was to investigate the FA composition of PL of spermatozoa, and to evaluate their fluidity through calculation of the mean melting point (MMP). The inverse measure of lipid fluidity can be calculated directly from the FA composition. In order to gain better insight in the physiological role of the sperm lipids, we determined the differences in FA composition of PL in two subpopulations of human spermatozoa present in normal and abnormal samples,

Table I. Semen sample profiles of the different groups. Results are given as the median with the 10–90th percentiles in parentheses

	Normozoospermia (n = 44)	Asthenozoospermia (n = 27)	Oligozoospermia (n = 12)
Sperm concentration (10^6/ml)	55.0 (26.5–95.0)	38.6 (21.9–76.7)	11.8 (2.9–17.9)
Grade (A+B) motility (%)	59.0 (47.4–72.2)	33.0 (15.0–46.0)	41.0 (26.0–48.9)
Linear velocity (μm/s)	27.2 (21.2–38.7)	19.5 (15.8–27.7)	19.9 (13.0–27.4)
Normal morphology (%)	22.0 (12.0–34.4)	14.0 (3.6–25.4)	6.0 (0.2–15.8)

separated by centrifugation on a discontinuous Percoll gradient (47 and 90%). In addition, we determined the FA composition of sperm heads and tails in an attempt to correlate this composition with the physiological function of these two regions.

Materials and methods

Semen samples (n = 83) from patients, consuming a freely selected diet, attending the andrology outpatient clinic of the university hospital of Ghent were included in the study. Semen samples were produced by masturbation and, after 30 min for liquefaction to occur, conventional semen analysis was performed according to the recommendations of the World Health Organization (WHO, 1992). Sperm concentration and motility were, however, assessed using a computer-assisted semen analysis (CASA) system (Autosperm; FertiPro, Lotenhulle, Belgium) (Hinting *et al.*, 1988). The differentiation of white blood cells (WBCs) and spermatogenic cells was performed by the peroxidase stain (Endtz, 1972) (LeucoScreen; FertiPro).

The semen samples were grouped into normozoospermic (n = 44), asthenozoospermic (n = 27), and oligozoospermic (n = 12) according to the recommendations of WHO (Rowe *et al.*, 1993), independent of the causal factor of the semen defect. Patients with oligo/asthenzoospermic semen samples included nine with varicocele, eight with male accessory gland infection (MAGI), and 22 with idiopathic infertility.

The spermatozoa were fractionated using a discontinuous Percoll gradient (47 and 90%) (PerWash; FertiPro). The Percoll gradients were centrifuged at 500 *g* for 20 min, the seminal plasma discarded, and cellular fractions were collected from the 47/90% interface (47% Percoll) and from the base of the gradient (90% Percoll) (Lessley and Garner, 1983; Aitken and Clarkson, 1988). The cells from each fraction were resuspended in 7 ml of Biggers–Whitten–Whittingham (BWW) medium (Biggers *et al.*, 1971) and centrifuged at 500 *g* for 5 min.

Fatty acid profile of the spermatozoa

The pellets of each fraction of Percoll were suspended in 1 ml of physiological saline and the lipids were extracted according to Folch *et al.* (1957). The lipids were separated by thin layer chromatography on rhodamine-impregnated silica gel plates using petroleum ether (bp 60–80°C; Merck Belgolabo, Overijse, Belgium)/acetone 85:15 as mobile phase (Christophe and Matthys, 1967). The PL band was scraped off and the FA converted into methyl esters by transesterification with 2.5 ml of a mixture of methanol: benzene: HCl (80:20:5) (Muskiet *et al.*, 1983). The methyl esters were extracted with petroleum ether (bp 40–60°C), evaporated to dryness under a N_2 flow at a temperature not exceeding 40°C, and analysed by temperature programmed capillary gas chromatography on a column of df polycyanopropyl (25 m×250 μm×0.2 μm) (Testec Corporation, Bellefonte, PA, USA) (Christophe *et al.*, 1992). Peak identification was performed by spiking with authentic standards (Sigma-Aldrich, Bornem, Belgium). Peak integration and calculation of the percent composition was performed electronically with a Varian Model 4290 integrator.

The oxidative potential index (OPI) of FA was estimated by summing the mole fraction of FA with 1, 2, 3, 4, 5, and 6 double bonds, multiplied by 1, 30, 70, 120, 180, and 240 respectively (Anttolainen *et al.*, 1996). The MMP of FA (sum of the mole fraction multiplied by the melting point for each FA) and the double bond index (DBI) (sum of mole fraction multiplied by the number of double bonds in the molecule for each FA) were calculated.

Higher unsaturated fatty acids (HUFA) were calculated by summing PUFA with at least 20 carbon atoms and three double bonds.

Preparation of head and tail fractions of spermatozoa

Spermatozoa recovered from the 90% Percoll fraction out of a pool of three semen samples of normospermic healthy donors, were resuspended in 2 ml Earle's medium and sonicated for 30 s (Soniprep 150; MSE, UK) at maximum power. The sonicated spermatozoa were examined microscopically to confirm the complete breakdown into heads and tails. The mixture of heads and tails was layered on top of a 90% Percoll column. After centrifugation (500 *g*; 20 min), the tails remained at the top of the column while the heads sedimented to the bottom. Each fraction was recovered, washed twice with 5 ml physiological saline, and the FA composition of the PL was determined. In order to validate the sonication and the separation of heads and tails procedures, in a separate experiment, the FA composition were determined of PL in spermatozoa, recovered from the 90% Percoll fraction, before and after sonication (no separation between heads and tails), and in a reconstituted sample in which the recovered head and tail fractions were mixed.

Statistical analysis

Statistical analysis was carried out using the MedCalc program (MedCalc Software, Mariakerke, Belgium) (Schoonjans *et al.*, 1995). Skewed distribution of some variables was normalized by square root or logarithmic transformation as appropriate, and the significance of differences was assessed by Student's *t*-tests. One-way analysis of variance (ANOVA) was used for overall comparison of independent groups, and the Student–Newman–Keuls test was used for pairwise comparisons. Correlation coefficients were calculated to test the relationship between variables (after data transformation if required).

Results

The semen profiles exhibited by the groups of this study are given in Table I. The percentages of oligozoospermic and asthenozoospermic semen samples with $>1\times10^6$ WBCs/ml were 33 and 20% respectively. The median value (10–90th percentile) of WBCs concentration of the infiltrated oligozoospermic samples was 2.6×10^6 ($1.1–4.3\times10^6$), and that of the asthenozoospermic samples was 2.5×10^6 ($1.2–6.5\times10^6$). The

Table II. Fatty acid composition (mean ± SEM) of the phospholipid fractions of spermatozoa recovered from 47% Percoll. Fatty acids are presented as mole percentage

	Normozoospermia (n = 44)	Asthenozoospermia (n = 27)	Oligozoospermia (n = 12)
14:0	1.96 ± 0.17^{c}	1.81 ± 0.21^{b}	1.67 ± 0.41
14:1ω5	0.42 ± 0.04^{c}	0.43 ± 0.04^{b}	0.45 ± 0.11
15:0	1.84 ± 0.39^{c}	2.24 ± 0.36	1.87 ± 0.39
16:0Br	2.68 ± 0.18^{a}	2.39 ± 0.30^{c}	2.04 ± 0.35^{b}
16:0	29.73 ± 0.56	29.84 ± 0.87	30.03 ± 1.14
16:1ω7	4.71 ± 0.42^{a}	4.12 ± 0.55^{a}	6.24 ± 0.99
17:0	1.09 ± 0.09^{a}	0.83 ± 0.10^{a}	0.73 ± 0.07^{b}
18:0Br	0.87 ± 0.13	0.77 ± 0.11	0.58 ± 0.07
18:0	11.35 ± 0.25^{a}	11.84 ± 0.34^{b}	13.35 ± 0.72**
18:1ω9	9.17 ± 0.47^{c}	8.71 ± 0.49^{b}	11.14 ± 1.06*
18:2ω6	3.91 ± 0.19^{a}	3.94 ± 0.12^{b}	4.41 ± 0.61
18:3ω6	0.35 ± 0.04^{b}	0.57 ± 0.08^{a}*	0.71 ± 0.17^{b}*
18:3ω3	0.17 ± 0.02^{b}	0.29 ± 0.06	0.45 ± 0.10**
20:0	0.77 ± 0.07^{b}	0.75 ± 0.09	0.63 ± 0.09
20:1ω9	0.65 ± 0.06^{b}	0.69 ± 0.08	0.71 ± 0.11^{b}
18:4ω3	0.23 ± 0.02^{c}	0.24 ± 0.03	0.29 ± 0.05
20:2ω6	0.61 ± 0.03^{c}	0.70 ± 0.05^{c}	1.06 ± 0.18**
20:3ω6	2.71 ± 0.35^{a}	2.64 ± 0.17^{a}	2.54 ± 0.16
20:4ω6	2.39 ± 0.14^{a}	2.88 ± 0.35^{b}	2.98 ± 0.41^{c}
22:0	0.66 ± 0.05	0.55 ± 0.10	0.53 ± 0.19
20:5ω3	0.48 ± 0.06	0.52 ± 0.08^{c}	0.61 ± 0.15
22:4ω6	0.47 ± 0.04	0.73 ± 0.11 *	0.81 ± 0.13**
24:0	0.33 ± 0.02	0.34 ± 0.03	0.35 ± 0.10
24:1ω9	0.96 ± 0.10	0.85 ± 0.12	0.68 ± 0.24
22:5ω3	1.64 ± 0.14	1.56 ± 0.26	1.31 ± 0.41
22:6ω3	21.54 ± 1.11^{a}	21.50 ± 1.49^{a}	15.98 ± 2.42^{b}*
SFA	49.95 ± 0.63^{a}	50.38 ± 0.96^{b}	51.24 ± 1.25
MUFA	15.49 ± 0.81^{a}	16.46 ± 0.94^{a}	19.17 ± 1.66*
PUFA	33.96 ± 1.26^{a}	33.82 ± 1.58^{a}	30.83 ± 2.76^{c}
ω6 HUFA	6.18 ± 0.30^{a}	7.14 ± 0.50^{a}	7.97 ± 0.64^{b}*
ω3 HUFA	24.36 ± 1.01^{a}	23.58 ± 1.34^{a}	18.07 ± 2.30^{b}**
Total ω6	10.09 ± 0.40^{a}	11.08 ± 0.52^{a}	12.38 ± 1.08*
Total ω3	24.45 ± 1.02^{a}	23.81 ± 1.35^{a}	18.45 ± 2.27^{c}*
ω6/ω3	0.45 ± 0.03	0.51 ± 0.04^{a}*	0.79 ± 0.13^{b}***
DBI	1.93 ± 0.05^{a}	1.90 ± 0.08^{a}	1.62 ± 0.13^{c}*
OPI	62.63 ± 2.72^{a}	62.61 ± 3.33^{a}	53.01 ± 5.20^{a}
MMP	20.04 ± 0.80^{b}	21.21 ± 1.15^{a}	25.77 ± 1.78^{c}**

Oligozoospermic and asthenozoospermic samples were significantly different from normozoospermic samples: *$P < 0.05$; ***$P < 0.01$; ****$P < 0.001$.
The superscript indicates the significance of difference as compared with the 90% Percoll sample:
$^{a}P < 0.001$; $^{b}P < 0.01$; and $^{c}P < 0.05$ (paired t-test).
SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids; HUFA = highly unsaturated fatty acids; DBI = double bond index; OPI = oxidative potential index; MMP = mean melting point.

Table III. Fatty acid composition (mean ± SEM) of the phospholipid fractions of spermatozoa recovered from 90% Percoll. The fatty acids are presented as mole percentage

	Normozoospermia (n = 44)	Asthenozoospermia (n = 27)	Oligozoospermia (n = 12)
14:0	2.47 ± 0.21^{c}	2.57 ± 0.28^{b}	2.26 ± 0.43
14:1ω5	0.56 ± 0.05^{c}	0.66 ± 0.05^{b}	0.67 ± 0.17
15:0	2.32 ± 0.50^{c}	2.62 ± 0.23	1.99 ± 0.26
16:0Br	1.75 ± 0.17^{a}	1.51 ± 0.19^{c}	1.12 ± 0.18^{b}
16:0	30.79 ± 0.44	29.94 ± 0.66	29.68 ± 1.20
16:1ω7	6.73 ± 0.39^{a}	7.36 ± 0.60^{a}	7.69 ± 0.92
17:0	1.72 ± 0.16^{a}	1.62 ± 0.08^{a}	1.54 ± 0.12^{b}
18:0Br	0.85 ± 0.06	0.83 ± 0.06	0.73 ± 0.07
18:0	12.18 ± 0.33^{a}	15.00 ± 1.13^{b}**	16.90 ± 2.72**
18:1ω9	10.22 ± 0.47^{c}	11.03 ± 0.61^{b}	12.39 ± 1.23*
18:2ω6	2.85 ± 0.11^{a}	3.32 ± 0.24^{b}*	4.40 ± 0.91**
18:3ω6	0.23 ± 0.03^{b}	0.26 ± 0.04^{a}	0.41 ± 0.14^{b}
18:3ω3	0.39 ± 0.08^{b}	0.43 ± 0.10	0.77 ± 0.21*
20:0	1.03 ± 0.10^{b}	0.89 ± 0.07	0.76 ± 0.17
20:1ω9	0.81 ± 0.06^{b}	0.86 ± 0.07	1.14 ± 0.17^{b}*
18:4ω3	0.28 ± 0.03^{c}	0.29 ± 0.02	0.36 ± 0.06
20:2ω6	0.49 ± 0.04^{c}	0.54 ± 0.06^{c}	0.83 ± 0.16**
20:3ω6	1.42 ± 0.08^{a}	1.57 ± 0.15^{a}	1.79 ± 0.23
20:4ω6	1.67 ± 0.16^{a}	2.07 ± 0.28^{b}	2.12 ± 0.27^{c}
22:0	0.82 ± 0.09	0.61 ± 0.09	0.48 ± 0.15
20:5ω3	0.54 ± 0.06	0.67 ± 0.09	0.76 ± 0.07*
22:4ω6	0.52 ± 0.04	0.68 ± 0.11	0.88 ± 0.13*
24:0	0.35 ± 0.06	0.36 ± 0.05	0.63 ± 0.22
24:1ω9	1.13 ± 0.13	0.88 ± 0.12	0.84 ± 0.21
22:5ω3	1.73 ± 0.14	1.39 ± 0.15	1.34 ± 0.21
22:6ω3	15.41 ± 1.09^{a}	10.09 ± 1.32^{a}**	8.92 ± 2.01^{b}**
SFA	52.05 ± 0.55^{a}	55.95 ± 1.09^{b}*	56.09 ± 2.63
MUFA	19.17 ± 0.83^{a}	20.79 ± 1.14^{a}	22.73 ± 1.88*
PUFA	25.62 ± 1.18^{a}	21.31 ± 1.52^{a}*	22.58 ± 1.64^{c}*
ω6 HUFA	4.59 ± 0.22^{a}	4.32 ± 0.46^{a}	4.79 ± 0.71^{b}*
ω3 HUFA	17.75 ± 1.07^{a}	12.15 ± 1.33^{a}**	11.02 ± 2.04^{b}**
Total ω6	7.25 ± 0.30^{a}	8.44 ± 0.52^{a}*	10.43 ± 1.19***
Total ω3	18.05 ± 1.06^{a}	12.87 ± 1.29^{a}**	12.15 ± 2.12^{c}**
ω6/ω3	0.48 ± 0.04	0.66 ± 0.11^{a}***	0.86 ± 0.52^{c}***
DBI	1.46 ± 0.07^{a}	1.18 ± 0.08^{a}*	1.19 ± 0.13^{c}*
OPI	45.98 ± 2.50^{a}	34.52 ± 3.19^{a}*	33.09 ± 5.06^{b}*
MMP	22.72 ± 0.68^{b}	28.95 ± 1.17^{a}*	30.31 ± 2.31^{c}***

Oligozoospermic and asthenozoospermic samples were compared with normozoospermic samples and significant differences were indicated with asterisks:
*$P < 0.05$; **$P < 0.01$; and ***$P < 0.001$.
The superscript indicates the significance of difference as compared with the 47% Percoll sample:
$^{a}P < 0.001$; $^{b}P < 0.01$; and $^{c}P < 0.05$ (paired t-test).
See Table II for abbreviations.

percentage of total cells recovered from 90% Percoll was not significantly different between the groups studied.

FA composition of the PL of normal and abnormal spermatozoa in the 47% and 90% Percoll fractions

PL and sterols were the only lipid classes present in the lipid extract of spermatozoa that could be identified and isolated by thin layer chromatography. The FA profiles of PL of spermatozoa recovered from the Percoll gradient (47 and 90%) fractions are given in Tables II and III. In normozoospermia, saturated fatty acids (SFA) made up 50.0 ±0.6% (mean ± SE, mole %) and 52.0 ± 0.6%, monounsaturated fatty acids (MUFA) comprised 15.5 ± 0.8% and 19.2 ± 0.8%, and polyunsaturated fatty acids (PUFA) represented 34.0 ± 1.3% and 25.6 ± 1.2% of total FA of PL in the 47% and 90% Percoll fractions respectively. The main SFA were 16:0 and 18:0, the main MUFA were 16:1ω7 and 18:1ω9, and the main PUFA was DHA which contributed >60% of total PUFA.

Significant differences in the FA composition of sperm PL were found in the oligozoospermic and asthenozoospermic samples when compared with normozoospermic samples. Total SFA were not significantly different in the 47% Percoll fraction between oligozoospermic and normozoospermic samples, but were significantly higher in the 90 % Percoll fraction of asthenozoospermic samples ($P < 0.05$). Only stearic acid (18:0) among SFA was significantly higher in oligozoospermic (47:90% Percoll, $P < 0.01$) and asthenozoospermic samples (90% Percoll, $P < 0.01$) compared with normozoospermic samples.

The proportions of total ω6 FA were higher in oligozoo-

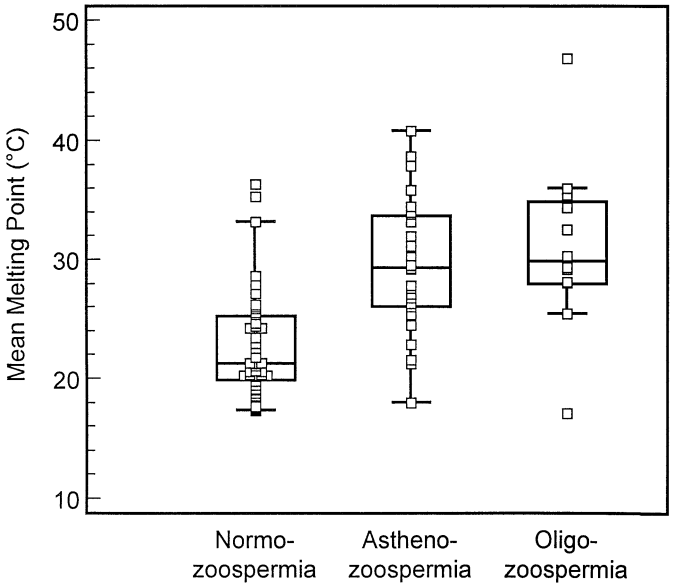

Figure 1. Box and whisker plots of the mean melting point (°C) of the fatty acids of phospholipids of spermatozoa recovered from 90% Percoll layer fraction of normozoospermic, asthenozoospermic, and oligozoospermic samples.

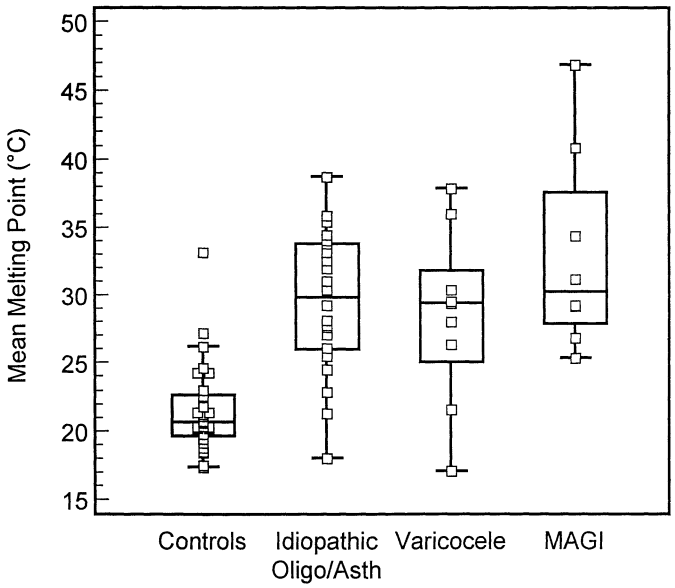

Figure 2. Box and whisker plots of the mean melting point (°C) of the fatty acids of phospholipids of spermatozoa recovered from 90% Percoll layer of patients with idiopathic oligo/astheno-zoospermia, varicocele, and male accessory gland infection (MAGI) compared with controls.

spermia (47% Percoll, $P < 0.05$; 90% Percoll, $P < 0.001$) and asthenozoospermia (90% Percoll, $P < 0.05$) than in normozoospermia. For individual ω6 FA, significant differences were seen in 18:2ω6 (90% Percoll), 18:3ω6 (47% Percoll), 20:2ω6 (47 and 90% Percoll), and 22:4ω6 (47 and 90% Percoll) with higher volumes in oligozoospermic samples then the controls. In contrast, total ω3 FA were lower in oligozoospermia (47% Percoll, $P < 0.05$; 90% Percoll, $P < 0.01$) and asthenozoospermia (90% Percoll, $P < 0.01$) than in normozoospermia. However, 18:3ω3 (47 and 90% Percoll), and 20:5ω3 (90% Percoll) were significantly higher in oligozoospermic samples. The most striking difference was the lower proportion of DHA (22:6ω3) in oligozoospermic (47% Percoll, $P < 0.05$; 90% Percoll, $P < 0.01$) and asthenozoospermic (90% Percoll, $P < 0.01$) samples than in the normozoospermic samples. The ω6/ω3 ratio was significantly higher in oligozoospermic (47% Percoll, $P < 0.001$; 90% Percoll, $P < 0.001$) and asthenozoospermic (47% Percoll, $P < 0.05$; 90% Percoll, $P < 0.001$) than in normozoospermic samples.

PUFA, DBI, and OPI were lower in oligozoospermic and asthenozoospermic samples (90% Percoll, $P < 0.05$) than in normozoospermic samples, and DBI was lower in the 47% Percoll fraction of oligozoospermic samples ($P < 0.05$).

The MMP was higher in oligozoospermia (47% Percoll, $P < 0.01$; 90% Percoll, $P < 0.001$) and asthenozoospermia (90% Percoll, $P < 0.05$) than in normozoospermia (Figure 1).

Differences between the 47 and 90% Percoll fractions

In the 90% Percoll fraction, SFA ($P < 0.0001$) and MUFA ($P < 0.0001$) of PL were higher than in 47% Percoll fraction. PUFA ($P < 0.0001$), DBI ($P < 0.0001$), and OPI ($P < 0.0001$) were lower in the 90% Percoll fraction, whereas the MMP ($P < 0.0001$) was higher. Total ω6 FA ($P < 0.0001$) and total ω3 FA ($P < 0.0001$) were lower in the 90% Percoll than in 47% Percoll fraction. The ω6/ω3 ratio was higher ($P < 0.01$) in the 90% Percoll layer of samples with abnormal sperm characteristics.

In normozoospermia, DHA represented 87 and 84% of the ω3 FA of the 47 and 90% Percoll fractions respectively. DHA of the 47% Percoll fraction of normozoospermic samples without WBCs was positively correlated with that of the 90% Percoll fraction ($n = 30$, $r = 0.91$, $P < 0.0001$). Total ω6 FA of 47 and 90% Percoll were also correlated ($r = 0.55$, $P < 0.0001$), as well as total ω3 FA ($r = 0.61$, $P < 0.0001$).

FA composition of the PL of spermatozoa recovered from the 90% Percoll of semen samples of patients with idiopathic infertility, varicocele, and male accessory gland infection(MAGI)

Table IV shows DHA, PUFA, total ω3 FA, ω3 HUFA, and DBI of PL of spermatozoa of the 90% Percoll layer to be significantly lower in patients with idiopathic oligo/asthenozoospermia, or MAGI than in the control group. In cases with varicocele, PUFA, total ω3 FA and ω3 HUFA, but not DHA and DBI, were lower than in the controls. The MMP of PL of spermatozoa recovered from the 90% Percoll of the three pathological groups were significantly higher than the control group (Table IV; Figure 2).

Relationship between FA and sperm characteristics

Table V shows significant correlations between motile sperm concentrations, grade (A+B), percentage motility, linear velocity, and proportion of normal sperm morphology on the one hand, and DHA, PUFA, ω3 HUFA, totalω3 FA, DBI, and MMP of spermatozoa recovered from the 90% Percoll layer on the other hand.

Table IV. Fatty acid composition (mean ± SEM) of the phospholipid fractions of spermatozoa recovered from 90% Percoll of different groups. Fatty acids are presented as mole percentage

	Control (n = 39)	Idiopathic oligo/ asthenozoospermia (n = 22)	Varicocele (n = 9)	Acessory gland infection (n = 8)
DHA	16.24 ± 1.16	10.93 ± 1.31*	10.32 ± 2.8	8.28 ± 2.12*
PUFA	26.84 ± 1.21	22.35 ± 1.54*	20.03 ± 2.78*	18.19 ± 2.09*
ω3 HUFA	19.07 ± 1.09	12.06 ± 1.45*	12.62 ± 2.84*	10.05 ± 1.90*
Total ω3	19.34 ± 1.11	12.59 ± 1.42*	12.97±2.86*	10.38 ± 1.97*
DBI	1.53 ± 0.06	1.21 ± 0.08*	1.19±0.19	0.99 ± 0.13*
MMP	21.51 ± 0.61	29.49 ± 1.11*	28.44±2.15*	32.95 ± 2.61*

*Idiopathic oligo/asthenozoospermia, varicocele, and acessory gland infection groups are significantly different from the control group ($P < 0.05$).
See Table II for abbreviations.

Table V. Correlations between fatty acid indices of spermatozoa recovered from the 90% Percoll fraction and sperm characteristics (n = 83) (r = correlation coefficient)

	Motile sperm concentration (10^6/ml)		Grade (A+B) motility (%)		linear velocity (μm/sec)		Morphology (% normal)	
	r	P	r	P	r	P	r	P
DHA	0.49	< 0.001	0.31	< 0.01	0.23	< 0.05	0.42	< 0.001
PUFA	0.43	< 0.001	0.34	< 0.01	0.18	= 0.12	0.39	< 0.01
ω3 HUFA	0.49	< 0.001	0.36	< 0.001	0.25	< 0.05	0.39	< 0.001
Total ω3	0.48	< 0.001	0.36	< 0.01	0.24	< 0.05	0.39	< 0.001
DBI	0.43	< 0.001	0.34	< 0.01	0.22	< 0.05	0.35	< 0.01
MMP	–0.50	< 0.001	–0.36	< 0.001	–0.31	< 0.01	–0.29	< 0.01

See Table II for abbreviations.

Differences between sperm heads and tails

The PL fractions of sperm heads contained a significantly higher proportion of DHA than that of sperm tails (Table VI). In addition, PUFA, total ω3 FA, OPI and the DBI were significantly higher in sperm heads than in sperm tails, but SFA, MUFA, and the ω6/ω3 ratio were lower in heads than in the tails. The mean MMP of the FA of the PL fractions of sperm heads was found to be 9.7°C lower than that of sperm tails. There were no differences of FA composition of PL of entire spermatozoa, sonicated spermatozoa (without separation of sperm heads and tails), or reconstituted mixture of heads and tails after sonication and Percoll separation (data not shown).

Discussion

The semen samples used in this study were fractionated on a discontinuous Percoll gradient to separate spermatozoa with high motility and competent function in the high-density solution (90% Percoll), from those with diminished motility and poor functional capacity in the low-density layer (47% Percoll). Previous studies have shown that spermatozoa from the 90% Percoll layer exhibit increased capacity for oocyte fusion in comparison with those of the lower density layer (Aitken and Clarkson, 1988). The percentage of total cells obtained in the two fractions of Percoll were not considered informative because we have studied the proportional FA composition of the phospholipids of spermatozoa.

In the present study, we have analysed the detailed FA composition of PL of spermatozoa, because PL are major structural components of membranes (Zaneveld *et al.*, 1991). The absence of other classes of lipids, e.g. triglycerides, cholesterol ester, free fatty acids, may be explained by the paucity of cytoplasm, due to its extension during sperm maturation. Also, the MMP was estimated as described by Holman *et al.* (1995) from all FA present, taking into account the degree of unsaturation, chain length, branching, and the change from solid to liquid state. The MMP is suggested to be related inversely to the fluidity of the membrane. We used OPI as an additional parameter which estimates the sensitivity to oxidation of fatty acids (Anttolainen *et al.*, 1996).

Our data demonstrate that most of the long chain metabolites of both linoleic acid (18:2ω6) and α-linolenic acid (18:3ω3) are present in human spermatozoa. Oleic acid (18:1ω9) and its metabolites, 20:1ω9 and 24:1ω9, were detected in human spermatozoa and in bovine and swine testes (Ahluwalia and Holman, 1969). Jones *et al.* (1979), and Alvarez and Storey (1992, 1995) have reported high amounts of DHA and the predominance of 16:0 and 18:0 among SFA of spermatozoa PL. These authors found relatively higher amounts of DHA in comparison with our results, which may be explained by differences in the methods of sperm preparation used to analyse the FA composition of PL.

The lower proportion of DHA, total PUFA, total ω3 FA, and DBI in spermatozoa from both Percoll fractions of oligozoospermic and in the 90% Percoll fraction of asthenozoospermic samples may be the result of excessive breakdown of PUFA due to increased generation of reactive oxygen species (ROS) in these samples (Aitken *et al.*, 1989; Zalata *et al.*,

Table VI. Fatty acid parameters of the phospholipid fractions of sperm heads and tails. The results are presented as means (95% confidence intervals) and the differences between the means (95% confidence intervals)

	Sperm heads	Sperm tails	Mean differences	*P*
DHA	33.67 (29.61–37.74)	14.04 (12.17–15.92)	19.62 (15.25–20.46)	< 0.0001
SFA	43.13 (39.27–46.97)	52.92 (51.33–54.51)	9.79 (5.64–13.9)	< 0.01
MUFA	9.11 (7.09–11.14)	19.52 (14.52–24.53)	10.41 (7.15–13.66)	< 0.01
PUFA	48.12 (41.13–55.10)	26.19 (20.06–32.33)	21.92 (20.54–23.31)	< 0.0001
Total ω6 FA	11.49 (8.36–14.63)	9.68 (6.32–13.03)	1.82 (–0.06–3.69)	$= 0.053$
Total ω3 FA	36.62 (32.66–40.59)	16.52 (13.57–19.47)	20.11 (18.81–21.39)	< 0.0001
ω6/ω3 ratio	0.31 (0.26–0.37)	0.58 (0.47–0.69)	0.27 (0.17–0.35)	< 0.01
DBI	2.59 1.44 (2.27–2.91)	1.15 (1.19–1.68)	< 0.0001 (1.06–1.24)	
OPI	93.97 (81.71–106.22)	45.21 (37.49–52.93)	48.76 (43.54–53.97)	< 0.0001
MMP	15.26 (12.61–17.92)	24.92 (23.88–25.97)	9.66 (7.13–12.19)	< 0.01

See Table II for abbreviations.

1995a). The increased ROS, and the resulting destructive effect on the PUFA of the PL, could be a consequence of chronic inflammation of the epididymis or prolonged exposure of spermatozoa to WBCs in the genital tract. In addition, this study and our previous data (Zalata *et al.*, 1995a) show an increased ratio of WBCs per 10^3 spermatozoa in oligo/asthenzoospermic samples, which could explain the deleterious effect of ROS in these samples. ROS was shown to be negatively associated with both the outcome of the sperm–oocyte fusion assay and with spontaneous pregnancy *in vivo* (Aitken *et al.*, 1991). Higher MMP in oligozoospermia and asthenozoospermia in comparison with normozoospermia corresponds with decreased fluidity of the sperm membrane. Hence, impaired fertility of men with oligo/asthenozoospermia could be due, at least in part, to the decreased fluidity of the sperm membrane. Indeed membrane fluidity regulates specific functions such as the acrosome reaction and fusion with the oocyte membrane. In addition, the significant increase of ω6/ω3 ratio in both oligozoospermic and asthenozoospermic, in comparison with normozoospermic samples, may suggest a physiological significance of this ratio because of specific interactions of ω6 and ω3 FA with certain membrane proteins and receptors (Lee *et al.*, 1986). However, the precise function of each FA of the sperm membrane still needs to be elucidated.

The FA composition of PL patterns of male accessory gland infection (MAGI), varicocele, and idiopathic oligo/asthenozoospermia suggest that destruction of PUFA, in particular of DHA, results in significant increase in the MMP. Patients with MAGI are more susceptible to the damaging effect of ROS produced by WBCs infiltration. Although the pathophysiological mechanisms involved in patients with varicocele, and the association between varicocele and MAGI are uncertain, the changes of the FA composition recorded in varicocele cases could explain, in part, the observed impairment of sperm quality.

The higher proportion of DHA, PUFA, total ω3 FA, and DBI in the spermatozoa recovered from the 47% Percoll fraction, which contains mostly immature spermatozoa, compared with those recovered from the 90% Percoll, may be related to a defect in the build-up of PL during the final stages of spermiogenesis and/or during epididymal sperm maturation. Indeed, other studies using whole rat spermatozoa and their purified plasma membranes have demonstrated that the concentration of PL and PL-bound FA declines progressively during sperm transit through different parts of the epididymal duct (Evans and Setchell, 1979; Parks and Hammerstedt, 1985). In addition, the ratio of saturated/unsaturated FA was shown to increase greatly during maturation of the sperm membrane. Our finding is contrary to the data of Hall *et al.* (1991). However, we have investigated the FA of PL of ejaculated human spermatozoa recovered by Percoll gradient, whereas Hall *et al.* (1991) investigated the membrane of rat spermatozoa from different parts of the epididymis. These differences may explain the discrepancy between results**.** The influence of contamination of 47% Percoll layer by WBCs on the FA composition of spermatozoa was believed to be small because of the low ratio of WBCs over spermatozoa. Also, the pattern of FA composition of PL of the 47% Percoll layer (rich in DHA) in comparison with the 90% Percoll suggest that there is no considerable influence of WBCs, which are poor in DHA (1.5%) (Griese *et al.*, 1990; Kelley *et al.*, 1993).

The FA profile of the mature spermatozoa recovered from the 90% Percoll may be related to the acquisition of sustained forward motility and the ability to bind to the zona-pellucida during the maturation process (Roberts, 1987). The native decapacitated state of ejaculated spermatozoa may be related to their relatively low proportion of PUFA and ω3 FA, particularly DHA, of PL. The capacitated state may be explained by removal of certain inhibitory factors and by modification of the lipid composition of the sperm membrane (Zaneveld *et al.*, 1991).

Spermatozoa recovered from the 47% Percoll fraction are more susceptible to oxidative stress than those from the 90% Percoll fraction since spermatozoa recovered from the former Percoll fraction have a higher OPI and, in addition, generate higher amounts of ROS (Zalata *et al.*, 1995a). Since a higher OPI is associated with increased unsaturation of the FA, our results could explain the finding of Alvarez and Storey (1995) that PUFA are more susceptible to oxidation than SFA during an experiment involving controlled aerobic peroxidation *in vitro*.

The strong correlation between DHA in spermatozoa recovered from the 47% and 90% Percoll fractions suggests that both subpopulations of spermatozoa recruit their FA from the same pool. It is not clear whether this pool can be affected either by differences in dietary intake (Christophe, 1996) or by differences in the FA transformation in testicular tissues. It has been shown that both Sertoli and germinal cells can synthesize 22:5ω6 and 22:6ω3 and that these reactions occur under hormonal control (Coniglio, 1994).

In order to prepare fractions of heads and tails, sonication was limited to a short time (30–60 s) followed by Percoll gradient fractionation. The sperm head consists mainly of condensed DNA and the acrosomal cap, and is covered by the

plasma membrane. The majority of PL are located in the membrane because of the paucity of cytoplasm. We did not investigate the influence of sonication upon the plasma membrane, but the fact that results from the isolated heads and tails are equivalent to these from the intact spermatozoa suggests that the membrane was not removed during this brief sonication period. The higher abundance of PUFA, total ω3 FA (especially DHA), and higher DBI of the PL of heads compared with that of tails found in this study is in disagreement with the results of Ahluwalia and Holman (1969) who found sperm tails to be richer in PUFA. However, the latter authors identified only trace amounts of DHA in the sperm heads and tails, in contrast to our results and to those of others (Alvarez and Storey, 1992; Lin *et al.*, 1993) suggesting methodological differences. The lower MMP of sperm heads than tails suggests that high fluidity of head membranes may be essential for the acrosomal reaction and oocyte fusion. It should be realized, however, that low MMP would be maintained if 20:4ω6 was substituted for DHA. Thus, DHA in human spermatozoa may have specific functions unrelated to fluidity, similar to such functions of DHA in the brain (Yamamoto *et al.*, 1987) and in the retina (Neuringer *et al.*, 1986).

In conclusion, the high content of unsaturated fatty acids particularly of DHA in spermatozoa, and the significant correlations between FA indices and DHA on the one hand, and sperm motility characteristics and morphology of spermatozoa recovered from 90% Percoll on the other hand, lend further support to the concept that the FA composition of PL of the sperm membrane has an important physiological role.

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