ORIGINAL ARTICLE

Decreased polyunsaturated and increased saturated fatty acid concentration in spermatozoa from asthenozoospermic males as compared with normozoospermic males

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Summary

The lipid composition of the sperm membrane has been shown to exert a significant effect upon the functional quality of spermatozoa. We have studied fatty acid composition of the phospholipids in spermatozoa in asthenozoospermic and normozoospermic men and determined the ratio of polyunsaturated fatty acids (PUFAs) to saturated fatty acids of spermatozoa of these two groups. Fatty acid concentration of spermatozoa was determined in 15 asthenozoospermic and eight normozoospermic semen samples by thin layer chromatography and gas chromatography. The most abundant polyunsaturated and saturated fatty acids in normozoospermic samples were docosahexaenoic acid (DHA 22 : 6 ω3, 98.5 ± 4.5 nmol per 10^8 spermatozoa, mean ± SE) and palmitic acid (103 ± 17 nmol per 10^8 spermatozoa) respectively. The mean ± SE values of DHA and palmitic acid in asthenozoospermic samples were 53.9 ± 11.6 and 145 ± 14.7 nmol per 10^8 spermatozoa respectively. Compared with normozoospermic samples, asthenozoospermic samples showed lower levels of PUFA and higher amount of saturated fatty acids. The mean ± SE ratios of sperm PUFA/saturated fatty acids in asthenozoospermic and normozoospermic samples were 0.66 ± 0.06 and 1.45 ± 0.16 ($P < 0.001$) respectively. This study demonstrates that spermatozoa of asthenozoospermic men have lower levels of PUFA compared with saturated fatty acids. This may be contributory to the poor motility noted in samples from these men.

Introduction

The sperm plasma membrane plays a very active role in the sperm fertilisation capacity and in sperm–oocyte fusion (Martinez & Morros, 1996; Flesch & Gadella, 2000). Its biochemical constitution is one of the main fields of interest in the study of sperm physiology and pathology (Lenzi *et al.*, 1996). Lipids play an important role in the functional activity of sperm and thus in male infertility (Gulaya *et al.*, 2001). Fatty acids, as a constituent of lipids in biological membranes, influence membrane properties such as fluidity, integrity, permeability, the activities of membrane-bound enzymes including the second messenger systems and membrane resistance to physical and chemical stress (Eder, 1995; Lenzi *et al.*, 2000b). Asthenozoospermia is considered to be contributory to the difficulty some men experience fathering children. In high percentages of these cases, the causes of decreased sperm motility are not completely understood (Cai & Marik, 1989).

In view of the importance of lipid structure, several studies have described the fatty acid composition of spermatozoa (Alvarez & Storey, 1995; Gulaya *et al.*, 2001; Lenzi *et al.*, 2000a; Zalata *et al.*, 1998a,b; Conquer *et al.*, 1999). The majority of available information on the fatty acid composition of spermatozoa is expressed by per cent

of mole or weight of fatty acids. However, the mole concentration of fatty acid-bound phospholipids in spermatozoa from asthenozoospermic samples has not been reported (Alvarez & Storey, 1995; Gulaya *et al.*, 2001; Lenzi *et al.*, 2000a; Zalata *et al.*, 1998a,b; Conquer *et al.*, 1999). Moreover, only one study (Alvarez & Storey, 1995) has reported the mole concentration of fatty acids from normozoospermic individuals.

The aim of the study was to investigate the fatty acid composition (mole concentration) of phospholipids from spermatozoa in normozoospermic and asthenozoospermic semen samples, and to evaluate the differences, if any, in the composition of fatty acids in normozoospermic and asthenozoospermic samples.

Materials and methods

Semen samples

Semen specimens were provided by 15 asthenozoospermic patients attending the Omid Fertility Clinic for infertility evaluation. In addition, eight normal fertile and healthy matching men with normal semen parameters according to World Health Organization (1999) criteria were enrolled as controls in this study. The two groups were similar with regard to mean age (20–40 years of age), smoking and drinking habits and had no systemic diseases. Patients fulfilling the inclusion criteria were asked to participate in this research project, which was duly explained to them. Written informed consent was obtained from all enrollees, according to the criteria of the Ethical Committee of Tehran University of Medical Sciences. All semen samples were collected by masturbation following 3 days of abstinence. After liquefaction, semen volume, sperm concentration (haemocytometer), total sperm count, morphology (Papanicolaou staining method) and motility grades [a (rapid progressive), b (slow progressive), c (nonprogressive), d (immotile)] were determined using World Health Organization (1999) standard procedures. All major determinations were carried out in duplicate. Semen samples with more than 1×10^6 ml^{-1} neutrophils (peroxidase staining; World Health Organization, 1999) or other round cells were excluded. Asthenozoospermia was indicated by a sperm concentration of $\geq 20 \times 10^6$ ml^{-1} and a motility (grade a + b) of <50%, irrespective of the morphology results. Normozoospermia was indicated by a sperm concentration of $\geq 20 \times 10^6$ ml^{-1} and a motility (grade a + b) of ≥50% and a normal morphology of ≥14%. Following semen analysis, a volume of semen containing at least 50 million spermatozoa was transferred into a conical centrifuge tube and was centrifuged at 1000 ***g*** for 10 min at room temperature. Immediately after the centrifugation, the supernatant was removed and the pellet from each sample was resuspended in 0.2-ml physiological saline (Gulaya *et al.*, 2001).

Lipid peroxidation in spermatozoa was measured by the reaction of thiobarbiuric acid with malondialdehyde (MDA) according toYagi, (1984).

Lipid extraction

Lipids were extracted from sperm using the Bligh & Dyer (1959). Methanol and chloroform were added to the sperm suspension with intermittent agitation for 15 min so that the ratio of methanol : chloroform : aqueous solution (sperm suspension) was 2 : 1 : 0.8. Additional water and chloroform were added so that the final ratio of methanol : chloroform: aqueous solution was 1 : 1 : 0.9. After centrifugation at 600 ***g*** for 5 min, the lower phase, which contained purified lipid, was aspirated. The upper phase was extracted once more in the same manner. The lower phase of the second extract was combined with the first extract and dried under a stream of nitrogen (N_2).

Thin layer chromatography and gas chromatography of fatty acids

The extracted lipids were separated by thin layer chromatography on a silica gel plate (Christophe & Matthijs, 1967). The solvent system was a mixture of hexane : diethyl ether : acetic acid (70 : 30 : 1.5, v/v/v) (Lenzi *et al.*, 2000a). The plates were maintained in an atmosphere of N_2. All phospholipids' band was scraped off and the fatty acid moieties of the phospholipids were identified as the methyl esters by gas chromatography. The methyl esters were prepared as follows: 0.3 ml of sodium methoxide 30% and 0.7 ml of methanol were added to glass tube provided with screw cap tops lined with Teflon, already containing the particular phospholipid excised from the dry plate and mixed for 5 min (Alvarez & Storey, 1995). The tubes were then placed in a heating block at 40 °C for 1 h. Following methanolysis, the tubes were removed from the heating block, allowed to cool to room temperature and neutralised with 0.1 ml of 1 M HCl. Then, 3 ml of *n*-hexane per millilitre of the reaction mixture was added and mixed for 10 min, and then centrifuged at 600 ***g*** for 5 min to allow separation of both liquid phases. The upper hexane layer was aspirated to a glass tube. This procedure was repeated twice (Alvarez & Storey, 1995). The hexane extracts were combined, evaporated to dryness under N_2 at room temperature and redissolved in 0.1 ml of methanol. The fatty acid methyl esters were separated and quantified by gas chromatographic analysis, using a polyethylene glycol capillary column (TR-FFAP) with flame ionisation detector. The

instrument was calibrated by the comparison of retention times of standard mixtures of methyl esters with methyl heptadecanoate as internal standard (Alvarez & Storey, 1995). The results are expressed as nmol fatty acids per 10^8 spermatozoa.

Statistical analysis

Owing to the fact that the sperm concentration, motility, morphology, MDA and various other semen parameters determined were not normally distributed, the Mann–Whitney *U*-test was applied to compare the asthenozoospermic and normozoospermic groups. For assessing sperm fatty acid, ratio of (PUFA)/Saturated and sperm count, a one-tailed two-independent sample *t*-test was used. Correlation between variables was assessed using nonparameteric Spearman's *r*.

Results

The main semen parameters for the asthenozoospermic and normozoospermic groups are illustrated in Table 1. The lipid compositions in the form of total phospholipid, saturated and PUFA in asthenozoospermic and normozoospermic samples are given in Table 2. The most abundant saturated fatty acids and PUFAs in the normozoospermic and asthenozoospermic samples were palmitic acid and docosahexaenoic acid (DHA) respectively. The mean ± SD ratios of PUFA (DHA, arachidonic acid, linolenic acid and linoleic acid) to saturated fatty acids (stearic acid, palmitic acid and myristic acid) of normozoospermic and asthenozoospermic samples were 1.45 ± 0.16 and 0.66 ± 0.06 respectively (Table 3). This ratio was significantly different between the two groups ($P < 0.001$). Polyunsaturated and saturated fatty acids in normozoospermic and asthenozoospermic samples were 54.7% and 30.6% (mol%) versus 35.3% and 56.9% respectively. Myristic acid was only detected in asthenozoospermic samples. In addition, palmitic acid was significantly higher in asthenozoospermic samples compared with normozoospermic samples ($P < 0.05$). The content of stearic acid and linoleic acid in the asthenozoospermic samples was 3.5 and 0.5 times that of the normozoospermic samples respectively ($P < 0.001$and $P < 0.05$ respectively). The concentration of linolenic acid, arachidonic acid and DHA decreased in the asthenozoospermic samples and the decrease in DHA in the asthenozoospermic samples was significant ($P < 0.01$). Generally, in asthenozoospermic compared with normozoospermic samples, the mole per cent of PUFA decreased and mole per cent of saturated fatty acid increased.

We observed high significant correlations between DHA concentration and motility grade a from normozoospermic samples ($r = 0.9$, $P < 0.001$). Table 4 shows significant correlations between concentration of DHA, linoleic acid and arachidonic acid with sperm motility from the two groups. In addition, we found a positive and significant correlation between DHA concentration and normal sperm morphology from asthenozoospermic and normozoospermic samples ($r = 0.65$, $P < 0.05$ and $r = 0.86$, $P < 0.01$ respectively).

Discussion

In the present study, we have analysed the fatty acid composition of phospholipid from spermatozoa, because phospholipids are the major structural components of membranes (Zaneveld *et al.*, 1991). Our data demonstrate that DHA, palmitic acid, oleic acid, linoleic acid, arachidonic acid, linolenic acid and stearic acid are present in human spermatozoa. The concentrations of DHA, palmitic acid and oleic acid of normozoospermic samples were similar to those reported by Alvarez & Storey (1995).

Table 1 Basic semen parameters (mean ± SD) from normozoospermic ($n = 8$) and asthenozoospermic ($n = 15$) samples

	Normozoospermic ($n = 8$)	Asthenozoospermic ($n = 15$)
Volume (ml)	3.58 ± 1.16	3.35 ± 1.16
Sperm concentration (10^6 ml^{-1})	119.3 ± 79.3	87.6 ± 25.2
Total sperm count (10^6 spermatozoa per ejaculate)	393.2 ± 252	297.9 ± 134.7
Normal morphology (%)	21.1 ± 5.6	8 ± 3.3***
White blood cell (10^6 ml^{-1})	0.66 ± 0.34	0.72 ± 0.47
Motility grade a[a] (%)	26.8 ± 7	5.1 ± 4.3***
Motility grade a + b[a] (%)	58.7 ± 9.9	27.5 ± 10.1***
Motility grade c + d[a] (%)	41.2 ± 9.9	72.4 ± 10.1***
Motility grade d[a] (%)	31.2 ± 9.9	57.2 ± 11.6***

[a]Grade of sperm movement according to World Health Organization (1999) criteria: a, rapid progressive; b, slow progressive; c, nonprogressive; d, immotile.
***$P < 0.001$.

Table 2 Fatty acid-bound phospholipid and malondialdehyde concentration (mean ± SE) in spermatozoa from normozoospermic ($n = 8$) and asthenozoospermic ($n = 15$) men

Fatty acid	Normozoospermic ($n = 8$)		Asthenozoospermic ($n = 15$)	
	nmol per 10^8 spermatozoa	mol%	nmol per 10^8 spermatozoa	mol%
Myristic acid	0	0	8.5 ± 5*	2.3
Palmitic acid	103 ± 17	30.8	145 ± 14.7*	40.6
Stearic acid	14 ± 2.4	4.2	50 ± 6.5***	14
Oleic acid	34 ± 12.2	10.2	43.4 ± 6.6	12.1
Linoleic acid	30 ± 7.5	9	15.8 ± 1.9*	4.4
Linolenic acid	25 ± 8.6	7.5	18 ± 5.5	5.5
Arachidonic acid	29.3 ± 4.3	8.7	20.1 ± 4	5.6
Docosahexaenoic acid	98.5 ± 4.5	29.5	53.9 ± 11.6**	15.1
Total fatty acids	333.8		354.7	
Malondialdehyde (nmol per 10^7 spermatozoa)	0.09 ± 0.004		0.14 ± 0.004*	

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Table 3 Ratio of polyunsaturated fatty acid (PUFA) (docosahexaenoic acid, arachidonic acid, linoleic acid, linolenic acid) to saturated fatty acids (stearic acid, palmitic acid, myristic acid) of spermatozoa in normozoospermic ($n = 8$) and asthenozoospermic ($n = 15$) men

Sample	Ratio of fatty acids	Normozoospermic ($n = 8$)	Asthenozoospermic ($n = 15$)
1	PUFA/saturated	2.16	0.25
2	PUFA/saturated	1.67	0.85
3	PUFA/saturated	1.6	0.33
4	PUFA/saturated	0.85	0.89
5	PUFA/saturated	1.2	0.74
6	PUFA/saturated	1.16	0.79
7	PUFA/saturated	1	0.59
8	PUFA/saturated	2	0.43
9	PUFA/saturated	–	0.48
10	PUFA/saturated	–	0.54
11	PUFA/saturated	–	0.87
12	PUFA/saturated	–	1.2
13	PUFA/saturated	–	0.8
14	PUFA/saturated	–	0.51
15	PUFA/saturated	–	0.79
	Mean ± SD	1.45 ± 0.16	0.66 ± 0.06*

*$P < 0.001$.

Table 4 Correlations between fatty acid-bound phospholipid of spermatozoa and sperm motility from normozoospermic and asthenozoospermic men ($n = 23$) (r = correlation coefficient)

Fatty acid	Motility grade a		Motility grade c + d	
	r	P	r	P
Linoleic acid	0.68	<0.01	−0.62	<0.01
Arachidonic acid	0.62	<0.05	−0.55	<0.05
Docosahexaenoic acid	0.54	<0.05	–	–
Myristic acid[a]	–	–	0.5	<0.05

[a]In asthenozoospermic samples

However, the concentrations of myristic acid and stearic acid were lower than those reported by these authors. In addition, the contents of linoleic acid, linolenic acid and arachidonic acid were higher than the levels reported by Alvarez & Storey (1995). Various factors such as the methods of sperm preparation used to analyse the fatty acid composition of phospholipids may contribute to the differences noted.

In the present study, the ratio of PUFA/saturated fatty acid in normozoospermic samples (Table 3) was higher than that in asthenozoospermic samples ($P < 0.001$) and the ratio differed by a factor of 2.16. This might be a novel way to explain various differences between the function of spermatozoa from normozoospermic and asthenozoospermic samples and further research is necessary. In this research, DHA was detected as the main PUFA in the human spermatozoa and phospholipid-bound DHA plays a major role in the sperm-membrane fluidity (Ollero *et al.*, 2000). It has been reported that PUFAs (i.e. DHA) are involved in bending and flexing of sperm flagellum, therefore, in sperm motility (Connor *et al.*, 1998). In addition, we found that sperm motility grades (grades a, a + b, c + d, d) and sperm MDA concentration in asthenozoospermic and normozoospermic males (Tables 1 and 2) were significantly different ($P < 0.001$ and $P < 0.05$ respectively). The differences noted may be partly due to the peroxidation of PUFA and particularly DHA (the highest PUFA level) in spermatozoa from asthenozoospermic samples. Hence, accompanied by higher MDA concentration in asthenozoospermic samples (Tavilani *et al.*, 2005), these changes may contribute to the lower motility observed in these samples. Storey (1997) reported an increased fatty acid lipid peroxidation in PUFA with two or more double bonds (two or more bis-allylic methylene groups) from spermatozoa. Jones *et al.* (1979) showed the spermicidal

effect of exogenous peroxidised fatty acid owing to its inhibition of sperm motility. All these findings may relate to the impact of low DHA concentration on decreased sperm motility. In addition, in asthenozoospermic and normozoospermic samples, a positive and significant correlation was observed between the concentration of DHA and normal sperm morphology($r = 0.65$, $P < 0.05$ and $r = 0.86$, $P < 0.01$ respectively). The correlation may reflect the possibility that the differences in fatty acid composition of phospholipids in two groups may be partly related to differences either in sperm metabolism or maturity.

Related to our findings, it is obvious that the phospholipid-bound PUFAs (particularly DHA) are necessary for membrane integrity and sperm function. Therefore, DHA removal from the sperm membranes during lipid peroxidation and, consequently, a decline in the ratio of PUFA/saturated fatty acids would be expected to lead to a decrease in sperm motility and perhaps morphology as noted in asthenozoospermic samples. Oxidation of phospholipid bound DHA has been shown to be the major factor that determines the motile life span of sperm *in vitro* (Jones *et al.*, 1979; Aitken *et al.*, 1993; Alvarez & Storey, 1995) and also results in acrosomal loss and DNA oxidation (Fraga *et al.*, 1991, 1996).

It seems that an alteration in the ratio of PUFA/saturated fatty acids of the sperm cell can be postulated as a common base for sperm pathology in many andrological cases. Further studies may prove necessary.

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