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Fatty acid composition of spermatozoa and immature germ cells

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A great deal of attention has recently been given to the essential role of polyunsaturated fatty acids (PUFA) of sperm membranes. We studied the fatty acid composition of the immature germ cells (IGC) and of the sperm populations separated by Percoll gradient in the ejaculate of normozoospermic patients. Fatty acid pattern was analysed by combined gas chromatography–mass spectrometry on a capillary column. In IGC, differences were found compared with mature spermatozoa, with a higher percentage of saturated fatty acids and of essential fatty acids. On the contrary, the long-chain PUFA were significantly lower in IGC. The highest concentration of *n*3 PUFA docohexaenoic acid (DHA) was detected in the spermatozoa deriving from 70–100% Percoll layers and a direct linear correlation was found between the increase of DHA and increased percentage of Percoll gradient. An inverse relationship between the percentage of atypical sperm forms in each layer and the percentage of DHA was also observed. This study demonstrates that the human germ cell line can elongate and desaturate essential fatty acids and that the percentage of long-chain PUFA is correlated with the normal morphology of sperm cells.

Key words: docohexanoic acid/immature germ cells/polyunsaturated fatty acids/spermatozoa/sperm morphology

Introduction

In recent years, a great deal of attention has been given to the sperm plasma membrane and its function during fundamental phenomena of the sperm life cycle, such as sperm maturation, sperm motility, acrosome reaction, and sperm–oocyte interaction. One of the major goals still to be reached in this field is the identification of the physiological plasma membrane constitution needed for normal sperm fertilizing ability; successful identification of this make-up could lead to its use as a marker of sperm function. This is not an easy task, bearing in mind the specific characteristics of spermatozoa swimming in semen; a pool of polarized cells at varying degrees of maturation, extremely susceptible to all external aggression and particularly to the induced peroxidation processes caused by reactive oxygen species (ROS) in the male and female genital tract secretions. This susceptibility is partly due to the relatively high concentration of polyunsaturated fatty acids (PUFA) of phospholipids (PL) in relation to the scarce quantity of cytoplasmic scavenging enzymes and partly to the equilibrium between anti/pro oxidant systems present in the sperm microenvironment (Alvarez *et al*., 1987, 1989; Aitken, 1991).

It is also important to note that current theories suggest that a prerequisite for normal cell–cell interaction and cell fusion is membrane fluidity, flexibility and ability to condition receptor expression and action. These characteristics are mainly dependent on sperm plasma membrane lipid constitution and the degree of PUFA unsaturation. Furthermore, the majority of the detectable PUFA in spermatozoa derive from the PL layers of their membranes because of the scarce quantity of cytoplasm. For all these reasons, over the last few years, PUFA have been studied as a marker of sperm pathophysiology. In normal subjects, a higher degree of unsaturation of sperm membrane PUFA was found compared with other cells of the body and differences between whole spermatozoa and Percoll-selected spermatozoa (Lenzi *et al*., 1996). Moreover, it has been reported that, in selected infertile patients with unilateral left varicocele and dyspermia, red blood cell plasma membranes show an alteration in the ratio between unsaturated and saturated fatty acids. This marker could therefore indicate an alteration in the general constitution of the cell membranes of these patients. Of the possible anti-oxidant therapies, glutathione has been able to produce an improvement in sperm motility and in the lipid peroxidation potential of semen (Lenzi *et al*., 1993, 1994) associated with an increase of the percentage of PUFA in the red blood cell membranes of these selected cases. In the present work, the fatty acid pattern of immature germ cells (IGC) and of sperm populations selected using modified Percoll gradients was studied.

Materials and methods

Donors and ejaculates

Healthy fertile donors (n = 10) were selected on the basis of the following seminal characteristics: sperm concentration 80×10^6 ml, forward motility >50%; atypical forms <45%. None of the subjects had been treated medically in the 6 months prior to the study. The

semen analyses were carried out according World Health Organization criteria (WHO, 1999). After the semen analysis the ejaculates were used for spermatozoa and IGC separation according to a modified Percoll gradient method (Berger *et al*., 1985), previously described (Gandini *et al*., 1999) and reported in the following paragraph. The percentage of atypical forms was evaluated in whole sperm sample and in the single Percoll layer containing selected spermatozoa. In the six ejaculates where the semen volume was >4 ml, the ejaculate was divided into two aliquots and the samples were processed using the two Percoll gradients reported below in order to obtain, in the same semen sample, the best selection of sperm populations and a careful isolation of IGC.

Percoll gradients

In order to have sperm populations selected on the basis of their morphology and motility as well as a careful isolation from leukocytes and IGC, an isotonic Percoll gradient (composed of the following layers/gradients 40–50–70–80–90–100%) was used. The method used was that of Berger (Berger *et al*., 1985), modified by the addition of a number of gradients, in order to obtain the best results (Gandini *et al*., 1999). In particular, regarding the separation of IGC, we used a Percoll gradient made up of 30–35–40–45–50–70–80–90–100% layers/gradients. Isotonic 100% Percoll (Sigma Chemical Co, St Louis, MO, USA) prepared by adding nine parts of Percoll to one part of Earle's salt solution 10× (Imperial, UK). Percoll was used in these procedures for experimental use only.

The procedures of separation were as follows: the isotonic Percoll 100% with Earle's salt solution (Imperial, UK) was diluted to obtain the dilutions 30, 35, 40, 45, 50, 55, 60, 70, 80, and 100%. The gradient columns (40–50–70–80–90–100% and 30–35–40–45–50–70–80–90–100% respectively) were prepared in a 15 ml Falcon tube by gently layering 1 ml of each of the above-mentioned solutions, starting from the 100% layer at the bottom. Semen (1 ml) was diluted with Earle's solution (1:2) and centrifuged at 400 *g* for 15 min at 18°C and the cell pellet was resuspended in 0.5 ml of Earle's solution. The semen cell suspension was gently stratified on top of the discontinuous Percoll gradient and centrifuged for 25 min at 800 *g* at 18°C. The single Percoll layers were separated and put into a single test tube.

The Percoll gradient for sperm population separation had the single layers analysed in order to evaluate sperm concentration, motility and morphology. Then each of them was mixed with Earle's solution (1:2) and centrifuged at 150 *g* for 10 min at 18°C. The Percoll gradient for IGC separation had the single layers analysed in order to select the ones with the greatest concentration of IGC. The layers which contain the majority of the IGC (generally the 30, 35, 40 and 45%) were pooled and diluted with Earle's solution (1:2) and centrifuged at 150 *g* for 10 min at 18°C. The pellet was resuspended in 1 ml of Earle's solution and the cell concentration evaluated.

IGC identification

May–Grünwald–Giemsa staining technique was used to identify various kinds of germ cells (spermatogonia, spermatocytes I and II, spermatids) and leukocytes (Schenck and Schill, 1988). As previously reported (Gandini et al., 1999), the separated IGC always showed normal morphology. The cell layers containing the majority of IGC were tested with an immunofluorescence microscopic technique using anti-CD45 fluorescent isothiocyanate (FITC) monoclonal antibodies (mAb) (El-Demiry *et al*., 1986). The leukocyte contamination was confirmed to be 1.5–6%, as previously demonstrated, using cytofluorimetry (Gandini *et al*., 1999). The vitality of the IGC isolated was evaluated using the eosin test (WHO, 1999).

Polyunsaturated fatty acids of sperm populations

In the whole semen cell samples, the single layers containing spermatozoa which had been selected on Percoll gradients and the pool of the layers containing the IGC were extracted twice in chloroform:methanol (2:1) (Merck, Darmstad, Germany) in the presence of butylated hydroxy toluene (BHT; Merck) (50 μg) as an antioxidant. The extracted lipids were separated by thin-layer chromatography on a silica gel plate. The solvent system was a mixture of hexane:diethyl ether:acetic acid (70:30:1.5, v/v/v). The phospholipids were scraped off from thin layer plates, extracted three times with 3 ml chloroform:methanol (1:1) and methylated for 30 s at 60°C in 30% sodium methoxide (Merck) in methanol. Tricosanoic acid (C23:0, 50 μg) was added as internal standard before scraping. The resulting methyl esters of fatty acids were analysed using a combined gas chromatography mass spectrometry (Hewlett Packard 5890 II gas chromatograph combined with 5989 mass spectrometry) system on capillary column (FFA-P, 60 m×0.32 μm×0.25 mm, Hewlett Packard). Helium was used as the carrier gas. Oven temperature from 80–200°C at 10°C /min and then to 230°C at 2°C/min was used. The results were obtained after time integration of chromatogram and final processing of the peak areas and are reported as a percentage of the total fatty acids analysed. The identification of each peak was checked comparing the mass spectrum with those of fatty acid methyl ester standards (Sigma).

Statistical analysis

Student's *t*-test was used to determine statistical significance of differences found in percentages of PUFA. The correlation that exists between the most representative PUFA and the sperm population selected by Percoll gradient was studied by the linear regression analysis (*r* values were calculated by Pearson test). The same correlation analysis was used between C22:6 *n*3 (docohexaenoic acid, DHA) and sperm morphology (% atypical forms). Graphical representations of the correlations were reported.

Results

Table I reports the seminal characteristics (pH, volume) the sperm parameters (concentration, forward motility and morphology) and the leukocyte concentrations in the 10 ejaculates were studied and IGC found in the six ejaculates with a volume of >4 ml. As the donors were healthy and fertile it must be noted that the data are in the normal range and, in particular, the leukocyte concentration was very low.

As reported in the second line of Table II, in whole spermatozoa it was found that the percentage of saturated fatty acids was 48–50% and palmitic (C16:0) and stearic acids (C18:0) were the most representative. Oleic acid (C18:1 *n*9) was the principal mono-unsaturated fatty acid (11.2 ± 1.7) and both the essential fatty acids linoleic (C18:2 *n*6) and linolenic acid (C18:3 *n*3) were present, but the concentration of the first was significantly higher (6.4 ± 1.4 and 0.33 ± 0.03). Among the desaturase metabolites, detectable concentrations were observed of C20:3, C20:4 and C22:4 belonging from *n*6 series and of C20:5 and C22:6 from *n*3 series. The total percentage of these PUFA was 33.56% and among these *n*6 PUFA represented 12.61% and *n*3 series 20.9%. Among the PUFA docohexaenoic acid (DHA, C22:6 *n*3) was the most representative (19.7 ± 8.4%).

Significant differences were found in the percentage distribution of fatty acids in the sperm populations and IGC separated

Table I. Seminal parameters in the 10 cases studied and concentration of the immature germ cells obtained after Percoll gradient procedure in the six cases with higher volume. Values are expressed as concentration of immature germ cells/ml of Earles' salt solution after separation

Case	pH	Volume ml	Sperm concentration $n \times 10^6$/ml	Forward motility (%)	Atypical forms (%)	Leukocytes $n \times 10^6$/ml	Germ cells $n \times 10^6$/ml *
1	7.3	4.3	75	40	40	0.50	1.5
2	7.5	4.0	100	55	48	0.40	1.0
3	7.7	4.0	140	60	38	0.70	1.3
4	7.5	4.4	150	50	45	0.70	1.4
5	7.4	4.1	120	60	38	0.55	2.1
6	7.5	4.3	70	55	48	0.30	1.8
7	7.4	2.8	110	55	46	0.60	
8	7.3	2.5	90	60	47	0.70	–
9	7.6	3.3	70	60	44	0.40	–
10	7.5	2,0	60	60	55	0.45	–

Table II. Pattern of fatty acids of plasma cell membranes from immature germ cells, whole sperm sample and sperm populations selected by Percoll gradient from healthy fertile donors. Results are reported as a percentage of each fatty acid in respect of the total fatty acids analysed by combined gas chromatography–mass spectrometry and represent the mean ± SD of the different samples

	C16:0	C18:0	C16:1 *n*7	C18:1 *n*9	C18:2 *n*6	C18:3 *n*3	C20:3 *n*6	C20:4 *n*6	C20:5 *n*3	C22:4 *n*6	C22:6 *n*3
Immature germ cells											
Mean	42.16	26.5	2.9	10	9.15	0.44	6.41	1.98	nd	nd	3.64
SD	1.4	4.27	0.5	0.42	1.67	0.04	2.43	0.5			1.53
Whole sperm sample											
Mean	29.32*	21.175	1.1	11.26	6.46*	0.33**	5.2	5.23**	1.22	2.18	19.7**
SD	7.94	0.4	0.08	1.76	1.45	0.03	1.96	0.3	0.003	0.5	8.4
Percoll-selected spermatozoa											
Fractions											
40 Mean	27.45	26.31	1.3	13.71	7.6	0.46	4.05	6.8	1.16	1.9	10.08
SD	1.33	10.7	0.05	2.4	2.5	0.025	0.29	2.05	0.2	0.2	2.32
50 Mean	32.09	20.18	1.1	8.19	6.29	0.38	6.14	5.2	1.22	2	16
SD	9.28	1.3	0.08	2.29	1.73	0.028	0.01	2.04	0.09	0.15	5
70 Mean	29	21.96	1.15	10.9	4.96	0.35	4.9	4.1	1.24	2	19.2
SD	1.69	5.39	0.1	9.15	2.43	0.028	1.91	0.75	0.25	0.2	3.02
80 Mean	29.5	27.8	0.9	4.1	3.93	0.3	4.11	4.08	1.7	2.2	19.93
SD	5.79	7.2	0.1	0.58	1.08	0.06	1.49	0.9	0.15	0.18	6.5
90 Mean	29	29.04	1	4.05	3.63	0.28	3.74	3.53	1.9	2.25	21.66
SD	6.5	3.34	0.12	0.96	0.63	0.028	0.97	0.71	0.18	0.28	5.4
100 Mean	30	25	0.8	3.63	4.17**	0.2**	4.25	4.5*	2.1**	2.7**	23.8**
SD	5.77	7.94	0.08	0.37	0.97	0.03	0.82	0.33	0.2	0.3	6.9

nd = not determined.
*$P < 0.005$; **$P < 0.0001$ with respect to the value of the germinal cells or fraction 40 respectively.

on Percoll gradient (Table II). The results are reported as a percentage of each fatty acid in respect to total fatty acids analysed and represent the mean ± SD of the different samples (six for IGC and 10 for whole spermatozoa and sperm populations selected by Percoll gradient).

In the sperm populations, the percentage of saturated fatty acids was not significantly modified whereas among the unsaturated fatty acids a significant rearrangement was observed. Evaluating linoleic acid (C18:2 *n*6) and α linolenic acid (C18:3 *n*3), the percentage of these essential fatty acids decreased from 7.6 ± 2.5 to 4.17 ± 0.9% and from 0.46 ± 0.025 to 0.2 ± 0.03% in the different Percoll fractions. Among the desaturase metabolites of the *n*6 series the percentage of C20:3 *n*6 was not significantly modified in the different Percoll fractions whereas the percentage of C20:4 *n*6 progressively decreased and this decrement was associated with an increase of C22:4 *n*6. On the contrary both the desaturase metabolites of the *n*3 series detected, C20:5 and C22:6 *n*3, progressively increased with the higher concentrations of Percoll gradient. Evaluating linoleic acid (C18:2 *n*6) and α linolenic acid (C18:3 *n*3), a significant inverse correlation with the Percoll gradient concentrations was observed ($r = -0.94$, $P < 0.005$, $r = -0.96$, $P < 0.001$, Figure 1a,b). On the contrary a direct linear correlation was found between the increase of DHA and those of the Percoll gradient ($r = 0.94$, $P < 0.005$, Figure 1c). The high percentage of C22:6 *n*3 was correlated with the morphology of the spermatozoa. In fact, an inverse relationship was observed ($r = -0.96$, $P < 0.005$) between the mean ± SD of the percentage of atypical forms scored in each layer, of the 10 ejaculates, and mean ± SD of percentages of C22:6 *n*3 evaluated in the same layers (Table III). The best morphological pattern corresponded to the highest content of this polyunsaturated fatty acid.

In respect to the pattern of mature cells, significant differ-

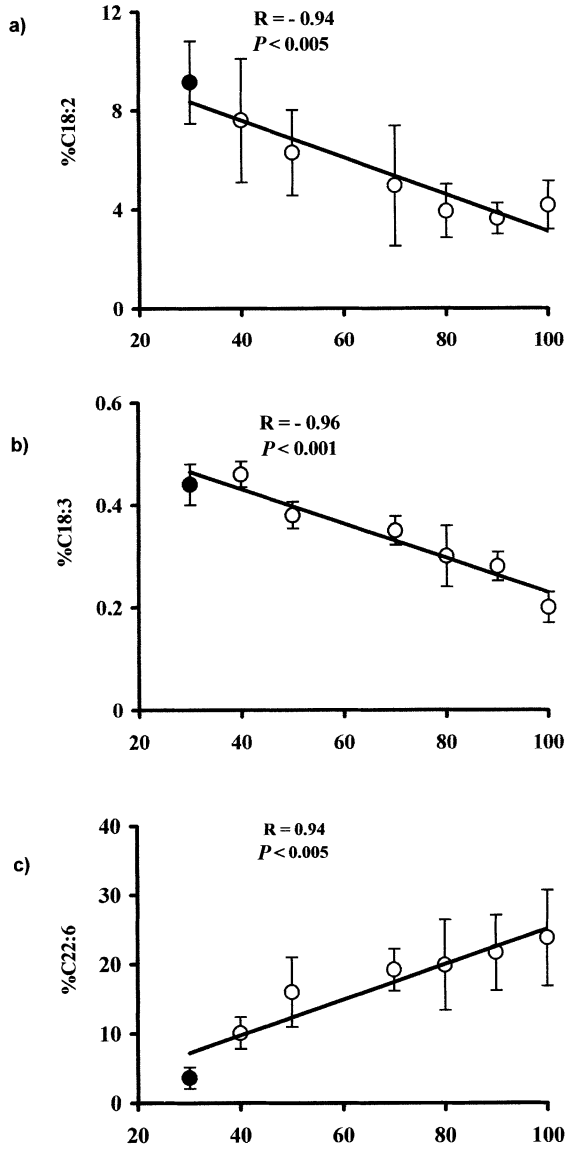

Figure 1. Linear correlation between the percentage of (**a**) C18:2 *n*6; (**b**) C18:3 *n*3 and (**c**) C22:6 *n*3 fatty acids, and Percoll-selected sperm populations (PSSP). Black dots represent the values for immature germ cells.

Table III. Percentage of atypical forms and percentage of fatty acid C22:6 *n*3 in sperm cell populations separated by Percoll gradient

Percoll-selected sperm fractions		Percentage atypical forms	Percentage C22:6 *n*3
40	mean	75.85	10.08
	SD	8.29	2.32
50	mean	64.57	16.0
	SD	4.23	5.0
70	mean	54.71	19.2
	SD	4.27	3.02
80	mean	48.57	19.93
	SD	4.5	6.5
90	mean	45.57	21.66
	SD	4.27	5.4
100	mean	43.14	23.8
	SD	3.48	6.9

$R = -0.98$.

ences were found in the IGC isolated by the second Percoll gradient (Table II). As in sperm cells, the saturated fatty acids were mainly represented by C16:0 and C18:0, but the percentage in respect to the total fatty acids analysed was significantly higher, being 68.66% ($P < 0.005$). Mono-unsaturated fatty acids, were composed of C16:1 *n*7 and C18:1 *n*9 and represented 12.9 ± 1.2% and the percentage of linoleic acid (C18:2 n6) was 9.1 ± 1.6% and of α linolenic acid (C18:3 *n*3) was 0.44 ± 0.04%, significantly higher than in mature sperm cells ($P < 0.005$ and $P < 0.0001$). On the contrary, among the desaturase metabolites *n*6 PUFA represented the 8.39% and *n*3 PUFA the 3.64%. These values were significantly lower in comparison with those observed in mature sperm cells. Di-homo y-linolenic acid (C20:3 *n*6), arachidonic acid (C20:4 *n*6) and docosahexaenoic acid (C22:6 *n*3) were the main PUFAs represented but they were present at different percentages compared with mature sperm cells, being 6.4 ± 2.4%;1.9 ± 0.5% and 3.6 ± 1.5% respectively ($P < 0.001$).

Discussion

The lipid components of sperm cell membranes derive from that of a spermatogonion that, during the various mitotic and meiotic divisions, receives metabolic contribution from intracellular synthesis processes. A complete rearrangement of the membrane structure during the sperm passage through the epididymis has been shown in animal models (Gaunt *et al*., 1983; Myles *et al*., 1984; Cowan *et al*, 1986; Wolf *et al*., 1986). It has also been shown that biomembrane fluidity and the degree of PUFA unsaturation increases when spermatozoa pass from the caput to the cauda of the epididymis (Hall *et al*., 1991). In rat, it has been shown that both long-chain *n*6 and *n*3 PUFA can be synthesized both in germinal and Sertoli cells under hormonal control (Coniglio, 1994), indicating an active sperm lipid metabolism of the testis including the desaturation and elongation of the essential fatty acids. Furthermore, these chances could be, in part, related to the loss of most cytoplasm along with a large loss of membrane lipids during the sperm maturation process. In this connection morphological, sperm membrane surface and enzymatic changes have been shown to be strictly related to the segregation of phospholipids (Huszar and Vigue, 1993, Huszar *et al*., 1997, Gergely *et al*., 1999) and an asymmetric transversal distribution of phospholipids has also been demonstrated in animal sperm membrane (Muller *et al*., 1994). Finally, a significant variability in lipid dynamics between membrane surface regions was also observed in human spermatozoa after cryopreservation, giving a possible reason for their reduced motility and fertilizing ability (James *et al*., 1999).

The results of this study show that human germ cell line have an active lipid metabolism which produces a rearrangement of the constitution of the fatty acids, causing an elongation and desaturation of the essential fatty acids during the spermatogenesis and possibly also during sperm maturation process. In fact, it was found that palmitic and stearic acid are the most representative saturated fatty acids in whole spermatozoa and that oleic acid was the most frequent mono-unsaturated one.

Of the essential fatty acids, the concentration of linoleic acid (C18:2 *n*6) was higher than that of α linolenic acid (C18: 3 *n*3) whereas, as recently reported (Lenzi *et al*., 1996; Zalata *et al*., 1998a), DHA was the most representative long-chain PUFA of mature sperm cells. On the contrary, in IGC the percentage of saturated fatty acids and of the essential fatty acids, linoleic and linolenic acids, was significantly higher whereas the percentage of the long-chain PUFA C20:4 *n*6, C20:5 *n*3, C22:4 *n*6 and C22:6 *n*3 were significantly lower.

Significant differences were found in the percentage of fatty acids in the sperm populations selected by Percoll gradient confirming quite similar results, in PUFA percentages, obtained by using the swim-up selection technique and radiolabelled fatty acid incorporation (Alvarez and Storey, 1995). In these experiments, the percentage of essential fatty acids progressively decreased with an increasing Percoll concentration and this decrease was associated with an increase of the content of C22:4 *n*6 and of C22:6 *n*3. The concentration of long-chain PUFA increased in the layers 70–100% of the Percoll gradient, hence in the cells with the best morphology. Considering the metabolic pathway of essential fatty acids, these results could indicate that long-chain PUFA are actively produced during the maturation of sperm cells after testicular release, or that the spermatogenic process produces spermatozoa with different constitutions of PUFA, or, finally, that the epididymal microenvironment can modify the relative percentages of the various PUFA in different sperm populations. The high concentration of carnitine in the human epididymis could support the hypothesis of a post-testicular metabolism of fatty acids as shown in other mammals (Wolf *et al*., 1986; Coniglio, 1994). However, a testicular production of spermatozoa with different structure and degree of maturation and PUFA unsaturation or a post-testicular peroxidation of long-chain PUFA can also be postulated.

The inverse linear correlation found between the percentage of atypical cells and the percentage of C22:6 *n*3, the most representative long-chain PUFA, indicates that the degree of PUFA unsaturation is related to normal sperm structure and function. Studies on the various methods of sperm selection have shown that spermatozoa from highly concentrated Percoll gradients (90–100%) are the best, both in the ability to undergo a normal acrosome reaction under ionophore stimulation and in the sperm–oocyte interaction (Aitken and Clarkson, 1988). However, the *n*3 PUFA, and particularly DHA, is present in other tissues devoted to signal transmission and cell to cell interaction such as the brain and retina (Neuringer *et al*., 1986; Yamamoto *et al*., 1987). It is possible to postulate that DHA has a similar role in spermatozoa and that part of these cells can show this specific constitution to enable the membrane fusion processes of capacitation and fertilization (Zaneveld *et al*., 1991).

However, this does not permit final conclusions regarding the pre- or post-testicular metabolism of PUFA in IGC and mature spermatozoa. In fact, the lipid composition of spermatozoa is also crucial in regulating the action of reactive oxygen species (ROS) on spermatozoa and they are active both in activating and in damaging the biomembrane. The harmful impact of oxyradicals on sperm function has been well studied (Aitken *et al*., 1987) and it has been correlated both with sperm pathologies and with in-vitro sperm function (Aitken *et al*., 1989; D'Agata *et al*., 1990; Twigg *et al*., 1998). In-vitro experiments have shown that cells isolated from the 40–65% Percoll gradients, consisting mostly of leukocytes and damaged or older sperm cells, are the main source of ROS (Aitken and Clarkson, 1988, Zalata *et al*., 1998b) whereas cells isolated at the bottom of the gradients, consisting of morphologically normal spermatozoa, do not produce ROS and have a higher *in vitro* fertilizing ability (Mortimer, 1991). These findings on the higher percentage of long-chain PUFA in morphologically normal spermatozoa selected from gradients from 70 to 100%, compared with those deriving from 40–50% gradients could indicate that ROS production can peroxidate long-chain PUFA (e.g. DHA) reducing its relative percentage in the most sensitive sperm populations.

In conclusion, these results demonstrate that the human germ cell line is able to metabolize lipids, in particular to synthesize and metabolize polyunsaturated fatty acids from IGC to mature spermatozoa and that the percentage of PUFA in sperm cells seems strictly related to their normal morphology and function.

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References

Aitken, R.J. (1991) Reactive oxygen species and human sperm function. In Bacetti, B. (ed.), *Comparative Spermatology 20 years After.* Serono Series. Vol. 75. Raven Press, New York, USA, pp. 787–792.

Aitken, R.J. and Clarkson, J.S. (1987) Cellular basis of defective sperm function and its association with the genesis of reactive oxygen species by human spermatozoa. *J. Reprod. Fertil.*, **81**, 459–469.

Aitken, R.J. and Clarkson, J.S. (1988) Significance of reactive oxygen species and antioxidant in defining the efficacy of sperm preparation techniques. *J. Androl.*, **9**, 367–376.

Aitken, R.J., Clarkson, J.S. and Fishel, S. (1989) Generation of reactive oxygen species, lipid peroxidation and human sperm function. *Biol. Reprod.*, **40**, 183–187.

Alvarez, J.G., Touchtone, J.C., Blasco, L., and Storey, B.T. (1987) Spontaneous lipid peroxidation and production of superoxide and hydrogen peroxide in human spermatozoa: superoxide dismutase as major protectant against oxygen toxicity. *J. Androl.*, **8**, 338–348.

Alvarez, J.G. and Storey, B.T. (1989) Role of glutathione peroxidase in protecting mammalian spermatozoa from loss of motility caused by spontaneous lipid peroxidation. *Gamete Res.*, **23**, 77–90.

Alvarez, J.G. and Storey, B.T. (1995) Differential incorporation of fatty acids into and peroxidative loss of fatty acids from phospholipids of human spermatozoa. *Mol. Reprod Dev.,* **42**, 334–346.

Berger,T., Marrs, R.T. and Moyer, D.L. (1985) Comparison of techniques for selection of motile spermatozoa. *Fertil. Steril.*, **43**, 268–273.

Coniglio, J.G. (1994) Testicular lipids. *Prog. Lipid. Res.*, **33**, 387–401.

Cowan, A.E., Primakoff, P. and Myles, D.G. (1986) Sperm exocytosis increases the amount of Ph-20 antigen on the surface of guinea-pig sperm. *J. Cell. Biol.*, **103**, 1289–1297.

D'Agata, R., Vicari, E., Moncada, M.L. *et al.* (1990) Generation of reactive oxygen species in subgroups of infertile men. *Int. J. Androl.*, **13**, 344–351.

El-Demiry, M.I.M., Hargreave, T.B. and Busuttil, A.A. (1986) Identifying leukocytes and leukocyte subpopulations in semen using monoclonal antibody probes. *Urology*, **6**, 492–496.

Gandini, L., Lenzi, A., Lombardo, F. *et al.* (1999) Immature germ cell separation using a modified discontinuous Percoll gradient technique in human semen. *Hum. Reprod.*, **14**, 1022–1027.

Gaunt, S.J., Brown, C.R. and Jones R. (1983) Identification of mobile and fixed antigens on the plasma membrane of rat spermatozoa using monoclonal antibodies. *Exp. Cell. Res.*, **144**, 275–284.

Gergely, A., Kovanci, E., Senturk, L. *et al.* (1999) Morphometric assessment of mature and diminished-maturity human spermatozoa: sperm regions that reflect differences in maturity. *Hum. Reprod.*, **14**, 2007–2014.

Hall, J.C., Hadley, J. and Doman, T. (1991) Correlation between changes in rat sperm membrane lipids, protein, and the membrane physical state during epididymal maturation. *J. Androl.*, **12**, 76–87.

Huszar, G. and Vigue, L. (1993) Incomplete development of human spermatozoa is associated with increased creatine phosphokinase concentration and abnormal head morphology. *Mol. Reprod Dev.*, **34**, 292–298.

Huszar, G., Sbracia, M., Vigue, L. *et al.* (1997) Sperm plasma membrane remodeling during spermiogenetic maturation in men: relationship among plasma membrane beta, 1,4-galactosyltransferase, cytoplasmic creatine phosphokinase, and creatine phosphokinase isoform ratios. *Biol. Reprod.*, **56**, 1020–1024.

James, P.S., Wolfe, C.A., Mackie, A. *et al.* (1999) Lipid dynamics in the plasma membrane of fresh and cryopreserved human spermatozoa. *Hum. Reprod.*, **14**, 1827–1832.

Lenzi, A., Culasso, F., Gandini, L. *et al.* (1993) Placebo controlled, double blind, cross-over trial of glutathione therapy in male infertility. *Hum. Reprod.*, **8**, 1657–1662.

Lenzi, A., Picardo, M., Gandini, L. and Dondero, F. (1996) Lipids of the sperm plasma membrane: from polyunsaturated fatty acids considered as markers of sperm function to possible scavenger therapy. *Hum. Reprod. Update*, **2**, 246–256.

Lenzi, A., Picardo, M., Gandini, L. *et al.* (1994) Glutathione treatment of dyspermia: effect on the lipoperoxidation process. *Hum. Reprod.*, **9**, 2044–2050.

Mortimer, D. (1991) Sperm preparation techniques and iatrogenic failures of in-vitro fertilization. *Hum. Reprod.*, **6**, 173–176.

Muller, K., Pomarski, T., Muller, P. *et al.* (1994) Protein-dependent translocation of aminophospholipids and asymmetric transbilayer distribution of phospholipids in the plasma membrane of ram sperm cells. *Biochemistry*, **33**, 9968–9974.

Myles, D.G. and Primakoff, P. (1984) Localized surface antigens of guinea pig sperm migrate to new regions prior to fertilization. *J. Cell. Biol.*, **99**, 1634–1641.

Neuringer, M., Connor, W.E., Lin, D.S. *et al.* (1986) Biochemical and functional effects of prenatal and postnatal omega-3 fatty acid deficiency on retina and brain in rhesus monkeys. *Proc. Natl Acad. Sci. USA*, **83**, 4021–4025.

Schenck, U. and Schill, W.B. (1988) Cytology of the human seminiferous epithelium. *Acta Cytol.*, **32**, 689–696.

Twigg, J., Fulton, N., Gomez, E. *et al.* (1998) Analysis of the impact of intracellular reactive oxygen species generation on the structural and functional integrity of human spermatozoa: lipid peroxidation, DNA fragmentation and effectiveness of antioxidants. *Hum. Reprod.*, **13**, 1429–1436.

World Health Organization (1999) *WHO Laboratory Manual for the Examination of Human Semen and Semen–Cervical Mucus Interaction.* Cambridge University Press, Cambridge, UK, pp. 4–23.

Wolf, D.E., Hagopian, S.S., Lewis, R.G. *et al.* (1986) Lateral regionalization and diffusion of a maturation-dependent antigen in the ram sperm plasma membrane. *J. Cell. Biol.*, **102**, 1826–1831.

Yamamoto, N., Saitoh, M., Moriuchi, A., *et al.* (1987) Effects of dietary alpha-linolenate/linoleate balance on brain lipid composition and learning ability of rats. *J. Lipid Res.*, **28**, 144–151.

Zalata, A.A., Christophe, A.B., Depuydt, C.E. *et al.* (1998a) The fatty acid composition of phospholipids of spermatozoa from infertile patients. *Mol. Hum. Reprod.*, **4**, 111–118.

Zalata, A.A., Christophe, A.B., Depuydt, C.E. *et al.* (1998b) White blood cells cause oxidative damage to the fatty acid composition of phospholipid of human spermatozoa. *Int. J. Androl.*, **21**, 154–162.

Zaneveld, L.J.D., Mack, S.R., Han, H.L. *et al.* (1991) Role of arachidonic acid metabolism in the human sperm acrosome reaction. In Bacetti, B. (ed.), *Comparative Spermatology 20 years After.* Serono series. Vol. 75. Raven Press, New York, USA, pp. 277–281.

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