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Relationship between iron-catalysed lipid peroxidation potential and human sperm function

R. J. Aitken, D. Harkiss and D. Buckingham

MRC Reproductive Biology Unit, 37 Chalmers Street, Edinburgh EH3 9EW, UK

The relationship between lipid peroxidation and the functional competence of human spermatozoa has been investigated in a cohort of 31 infertility patients. Lipid peroxidation was assessed using a sensitive fluorometric assay for the generation of malondialdehyde in response to the presence of a ferrous ion promoter. Sperm function was evaluated by monitoring the movement characteristics of these cells and their capacity for sperm–oocyte fusion. Each sample was separated into high- and low-density sperm populations on discontinuous, two-step (40%:80%), Percoll gradients prior to analysis. The way in which individual ejaculates fractionated on these gradients was highly positively correlated ($P < 0.001$) with the lipoperoxidation status of the spermatozoa; the greater the potential for malondialdehyde generation, the higher the proportion of cells entering the low density region of the gradients. The lipoperoxidation potential of the freshly prepared spermatozoa was also highly predictive ($P = 0.0001$) of their capacity for movement at 3 and 24 h and their ability to exhibit sperm–oocyte fusion in response to the ionophore A23187. The potential for malondialdehyde generation in the 40% and 80% Percoll fractions was positively associated with midpiece abnormalities in the spermatozoa. These results emphasize the importance of lipid peroxidation in the pathophysiology of male infertility and suggest a mechanism by which such damage might arise.

Introduction

A significant new development in the field of male infertility has been the realization that oxidative stress can play a key role in the aetiology of this condition (Aitken and Clarkson, 1987a, b; Aitken *et al.*, 1989a, b, 1991, 1992a, b, 1993). The generation of reactive oxygen species by human sperm suspensions has been shown to correlate with the fertilization potential of human spermatozoa *in vitro* (Aitken and Clarkson, 1987a, b) and, in long term prospective studies, *in vivo* (Aitken *et al.*, 1991). The reactive oxygen species responsible for this oxidative stress appears to derive from the excessive activity of a superoxide-generating system located in the sperm plasma membrane (Aitken *et al.*, 1992b; Alvarez *et al.*, 1987; Iwasaki and Gagnon, 1992). Leucocytes, particularly polymorphs, also represent a powerful potential source of reactive oxygen species (Aitken and West, 1990; Kessopoulou *et al.*, 1992) which may be clinically significant in cases of leucocytospermia or during the preparation of spermatozoa for *in vitro* fertilization (Aitken and Clarkson, 1988). Experiments involving the generation of toxic oxygen metabolites in a xanthine oxidase system have established that the major reactive oxygen species responsible for damaging spermatozoa is hydrogen peroxide (Aitken *et al.*, 1993). The way in which this oxidant interferes with normal human sperm function is thought to involve the peroxidation of unsaturated fatty acids in the sperm plasma membrane (Jones *et al.*, 1978, 1979). If this is the case, then there should be a correlation between the incidence of defective sperm function and the peroxidation status of human spermatozoa.

The most convenient, and widely used, assay of lipid peroxidation is the thiobarbituric acid reaction for malondialdehyde, a small carbonyl compound which is produced as an end product of lipid peroxidation, probably from cyclic peroxides or endoperoxides. The generation of malondialdehyde can be significantly enhanced by the addition of a ferrous ion promoter to the incubation system (Jones *et al.*, 1979). Comaschi *et al.* (1989) and Aitken *et al.* (in press) have demonstrated that ferrous ions catalyse the breakdown of pre-existing lipid hydroperoxides in spermatozoa and the subsequent propagation of a lipid peroxidation chain reaction through the generation of peroxyl and alkoxyl radicals. Whether the degree of polyunsaturated fatty acid degradation observed under such circumstances is reflective of the functional competence of a given sperm population is an important clinical question which we have addressed in the present study.

Materials and Methods

Spermatozoa

Human spermatozoa were provided by a cohort of 31 patients whose infertility status was unknown, although major abnormalities in the female partner had been ruled out on the basis of normal history and examination, normal luteal phase

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progesterone concentrations and normal laparoscopy including the demonstration of tubal patency (Aitken *et al.*, 1991). The study was approved by the local Ethics Committee and the consent of the patients was obtained.

A conventional semen profile was obtained using the procedures laid down by the World Health Organization (1987) including the collection of data on semen volume and appearance, sperm concentration and total count, percentage motility and morphology. The latter was scored on wet preparations, immobilized by sperm diluting fluid (50 g $NaHCO_3$, 10 ml formalin in 1 l distilled water; World Health Organization, 1987) by phase contrast microscopy using a Leitz Ortholux microscope. The percentage of abnormal spermatozoa was determined by counting 200 cells, using the criteria of normality defined by the World Health Organization (1987), at a magnification of ×312.5. Deviations from the normal morphology defined by the World Health Organization (1987) were classified using a hierarchical system in which head, midpiece and tail defects were scored in succession. The presence of leucocytes and precursor germ cells in the ejaculates was determined using the techniques described by Aitken and West (1990) in which the leucocyte population was differentiated using a monoclonal antibody directed against the common leucocyte antigen (anti-CD45, Scottish Antibody Production Unit, Carluke) using an alkaline phosphatase, anti-alkaline phosphatase immunocytochemical technique.

The spermatozoa were isolated by discontinuous Percoll gradient centrifugation (Aitken and Clarkson, 1988) using a simple two-step design incorporating 40% and 80% Percoll, respectively. Isotonic (100%) Percoll was created by supplementing 10 ml of 10 × concentrated medium 199 (Flow Laboratories, Irvine) with 300 mg BSA, 3 mg sodium pyruvate and 0.37 ml of a sodium lactate syrup and adding 90 ml of Percoll (Pharmacia, Uppsala). This preparation was designated 100% Percoll and was subsequently diluted with HEPES-buffered medium BWW (Biggers *et al.*, 1971; Aitken and Clarkson, 1988) supplemented with 0.3% BSA. After an initial centrifugation at 500 ***g***, for 20 min the spermatozoa at the 40–80% interface (low density population) and at the base of the 80% Percoll fraction (high density population) were collected, washed with 5 ml BWW, and resuspended in BWW at a concentration of 2×10^7 ml^{-1}.

Hamster oocyte penetration test

The isolated spermatozoa were diluted 1:1 with 2.5 μmol A23187 l^{-1} formulated as the free acid and maintained as a 100 mmol l^{-1} stock solution in dimethyl sulfoxide (DMSO) at −20°C. A working solution was created by diluting this stock to 1 mmol l^{-1} with medium BWW and storing this material at 4°C for 3–30 days. The spermatozoa were incubated with the ionophore for 3 h at 37°C, in an atmosphere of 5% CO_2 in air, pelleted by centrifugation at 500 ***g*** for 5 min, resuspended in the same volume of fresh medium BWW and distributed as 50 μl droplets under liquid paraffin. Zona-free hamster oocytes were prepared as described by Yanagimachi *et al.* (1976) and dispensed into the droplets at five oocytes per drop and 15–20 oocytes per sample.

After a further 3 h, the oocytes are recovered from the droplets, washed free of loosely adherent spermatozoa, compressed to a depth of about 30 μm under a 22 × 22 mm coverslip on a glass slide and assessed for the presence of decondensing sperm heads with an attached or closely associated tail, by phase contrast microscopy. The number of spermatozoa penetrating each egg was assessed and, in view of the high rates of sperm–oocyte fusion observed, the results were expressed as the mean number of spermatozoa penetrating each oocyte (total number of penetrations/total number of oocytes) (Aitken and Elton, 1984). With every hamster egg penetration test, a positive control sample from a fertile donor was included. During this study ten controls were used; these samples gave a penetration rate of 100% in every instance and a mean sperm per oocyte score of 10.9 ± 1.3.

Motility

The percentage of motile spermatozoa was counted at × 100 with the aid of a grid on an eye piece graticule, and the movement characteristics were assessed using the European (25 Hz) version of the Hamilton Thorn motility analyser (Version 7) at a temperature of 37°C using the following settings: minimum contrast, 12; minimum size, 3; low and high size gates, 0.4 and 1.6, respectively; low and high intensity gates, 0.5 and 2.0, respectively; nonmotile head size, 8; nonmotile intensity, 201; per cent rapid represented cells moving at > 25 μm s^{-1} while per cent progressive equated with a straightness (mean progressive velocity/mean path velocity) of > 75%. The samples were held in a Makler chamber (Sefi Medical Instruments, Haifa) for analysis.

Lipid peroxidation

Lipid peroxidation was measured using an optimized (Aitken *et al.*, in press) version of the thiobarbituric acid (TBA) test (Placer *et al.*, 1966) incorporating a fluorometric end-point (Yagi, 1976) that permitted the recovery and measurement of much greater concentrations of malondialdehyde than in previous assays (Aitken *et al.*, 1989a). The sperm suspensions in medium BWW were pelleted by centrifugation (500 ***g*** for 5 min) and resuspended at 10^7 ml^{-1} in Hanks balanced salt solution, lacking calcium and magnesium. Solutions of ferrous sulfate (4 mmol l^{-1}) and sodium ascorbate (20 mmol l^{-1}) were then prepared in distilled water and 10 μl of each stock solution added to 1 ml of sperm suspension to promote lipid peroxidation. The cells were incubated with the promoter for 2 h at 37°C. A TBA assay mixture was made up fresh each day comprising: 200 μl 7% SDS, 2 ml 0.1 mol HCl l^{-1}, 300 μl 10% phosphotungstic acid, 100 μl butylated hydroxytoluene and 1 ml 0.67% 2-thiobarbituric acid. Incubated sperm suspension, 200 μl, was then added to 360 μl of assay mixture and placed in a boiling water bath for 30 min. The tubes were cooled and the malondialdehyde–TBA adduct extracted by the addition of 500 μl of butanol for measurement in a spectrofluorimeter using excitation and emission wavelengths of 510 and 553 nm, respectively. Standards were prepared fresh for each assay by the overnight acid hydrolysis of 1,1,3,3,-tetraethoxypropane in 0.1 mol HCl l^{-1} (Sinnhuber and Yu, 1958). The results were expressed as nmol malondialdehyde generated 10^{-8} spermatozoa h^{-1}.

Table 1. Semen characteristics for the subjects used in this study

Total number of spermatozoa (10^6 per ejaculate)	Concentration (10^6 ml^{-1})	Motility (%)	Morphology (%)
405.6 ± 64.2	102.1 ± 14.1	59.1 ± 3.2	34.8 ± 3.1

Statistical tests

The data were transformed [log(1 + x)] to normalize the distribution of the data prior to the calculation of linear regression coefficients and *t*-tests using the STATVIEW statistical package (Abacus Concepts, Berkeley, CA) on an Apple Macintosh SE/30 computer.

Results

Semen quality

The population of donors used in this study exhibited the mean semen characteristics shown in Table 1. One specimen was slightly oligozoospermic (17.8×10^6 ml^{-1}), whereas seven were mildly asthenozoospermic (<40% motility), although none of the original semen samples gave a motility of less than 20%. Morphology was more severely affected in this cohort of subjects; 12 exhibited teratozoospermia (<40% normal morphology) and seven gave a normal morphology value of less than 20%. None of the samples exhibited leucocytospermia (>10^6 leucocytes ml^{-1} semen): the mean number of CD45-positive leucocytes was $6.31 \pm 2.75 \times 10^4$ leucocytes ml^{-1} semen.

Percoll fractionation

A total of 31 semen samples were processed in the course of this analysis, each of which generated two populations of cells, from the 40–80% interface (low density population) and the base of the 80% fraction (high density population), respectively. A significant ($P < 0.001$) difference was observed in the amount of malondialdehyde produced by these two populations of spermatozoa on exposure to the ferrous ion promoter. The cells recovered from the 40–80% interface generated three times more malondialdehyde than did those pelleting to the base of the 80% fraction (12.8 ± 2.1 versus 4.4 ± 1.1 nmol 10^{-8} spermatozoa h^{-1}). The enhanced susceptibility to lipid peroxidation exhibited by the sperm populations recovered from the 40–80% interface was associated with significant reductions in the percentage of morphologically normal spermatozoa (54.5 ± 4.1 versus 23.0 ± 2.1%; $P < 0.001$) and the percentage of motile cells (71.5 ± 4.1 versus 40.1 ± 3.2%; $P < 0.001$), measured within 15 min of preparing the spermatozoa.

The response of each ejaculate to Percoll gradient centrifugation was also highly correlated with the capacity of the spermatozoa to generate malondialdehyde in the presence of a ferrous ion promoter. Hence, the proportion of the total recovered sperm population which localized in the low density fraction (total number of cells recovered in the 40% fraction/the total number of cells recovered in the 80 + 40% fractions) was significantly and positively correlated with the potential of these cells for malondialdehyde generation ($r = 0.628$; $P < 0.001$; Fig. 1a). Furthermore, the proportion of the total sperm population migrating to the base of the high density 80% Percoll fraction was significantly and inversely related to the potential of these cells for malondialdehyde generation ($r = -0.516$; $P < 0.001$; Fig. 1b).

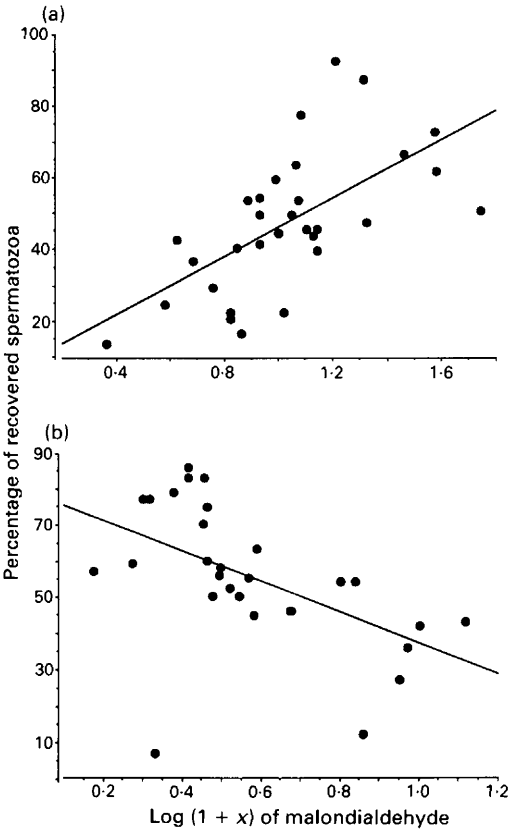

Fig. 1. Relationship between the generation of malondialdehyde and the proportion of the recovered human sperm population migrating to (a) the 40–80% Percoll interface and (b) the base of the 80% Percoll fraction.

Morphology

Analysis of the relationship between sperm morphology and the potential for malondialdehyde generation showed that there were significant ($P < 0.03$) correlations with abnormalities in the structure of the midpiece ($r = 0.401$ and $r = 0.400$ for the 80% and 40% fractions, respectively), but not the head or flagellum of the spermatozoa.

Movement characteristics after 3 h incubation

After isolation of the spermatozoa on the Percoll gradients, they were incubated in medium BWW at 37°C, in an atmosphere of 5% CO_2 in air, at a concentration of 10^7 ml^{-1} for 3 h. At the end of this period the movement characteristics of the spermatozoa were assessed and related to their capacity to

Table 2. Movement characteristics of human spermatozoa after 3 h incubation

Criterion	40%–80% Percoll interface	80% Percoll
Percentage rapid	14.7 ± 1.3	57.0 ± 4.4*
Percentage motile	24.7 ± 2.2	67.5 ± 4.2*
Mean path velocity ($\mu m\ s^{-1}$)	37.1 ± 1.3	58.7 ± 2.4*
Mean progressive velocity ($\mu m\ s^{-1}$)	30.06 ± 1.3	50.1 ± 2.3*
Mean curvilinear velocity ($\mu m\ s^{-1}$)	51.4 ± 1.8	80.25 ± 3.1*
Beat frequency (Hz)	10.9 ± 0.3	12.7 ± 0.2*
Amplitude of head displacement (μm)	3.6 ± 0.2	4.8 ± 0.2*
Progressive motility (%)	15.3 ± 1.4	47.6 ± 3.4*

*$P = 0.001$ (40% versus 80% Percoll fraction).

generate malondialdehyde in response to the presence of promoter. The high levels of lipid peroxidation observed in the 40% Percoll fraction were associated with significant reductions ($P < 0.001$) in a number of criteria of sperm movement relative to the 80% Percoll fraction, including the percentage of rapid cells, percentage motility, mean path velocity, progressive velocity, curvilinear velocity, beat frequency, amplitude of lateral head displacement and percentage of progressive cells (Table 2). Conversely, the proportion of slow spermatozoa exhibiting velocities of $< 10\ \mu m\ s^{-1}$ was significantly greater in this population of cells ($P < 0.01$) than in the 80% Percoll fraction.

Considering the data base as a whole (40 + 80% fractions), powerful ($P < 0.001$) negative, linear correlations were found between the generation of malondialdehyde and sperm movement at 3 h (Fig. 2) giving the following correlation coefficients: percentage of rapid cells ($r = -0.757$), percentage motility ($r = -0.775$), mean path velocity ($r = -0.664$), mean progressive velocity ($r = -0.596$), mean curvilinear velocity ($r = -0.660$), beat frequency ($r = -0.459$), amplitude of lateral head displacement ($r = -0.407$) and percentage progressive motility ($r = -0.708$; Fig. 2).

If the analysis was confined to the more clinically relevant, high density, 80% Percoll fractions (Fig. 3) then powerful ($P < 0.001$), negative linear correlations were again observed between the lipoperoxidation potential of the cells and their capacity for movement. In such samples the linear regression coefficients recorded were: percentage of rapid cells ($r = -0.826$), percentage motility ($r = -0.806$), mean path velocity ($r = -0.651$), mean progressive velocity ($r = -0.498$), mean curvilinear velocity ($r = -0.655$) and percentage progressive motility ($r = -0.631$). The proportion of slow moving cells was also positively correlated ($r = 0.600$; $P < 0.001$) with malondialdehyde generation.

Movement after 24 h

Incubation of the spermatozoa for 24 h in medium BWW at 37°C was associated with significant reductions in the percentage of motile cells and the various measurements of sperm head velocity in the 40 and 80% fractions (Tables 2 and 3). However, at the end of the incubation period significant differences were still apparent between the two Percoll fractions with respect to the percentages of motile, rapid and progressive spermatozoa (Table 3).

The ability of the individual sperm populations to sustain their motility over the 24 h incubation was accurately predicted by the generation of malondialdehyde in a separate aliquot of spermatozoa exposed to the ferrous ion promoter in calcium- and magnesium-free Hanks balanced salt solution for the first 2 h (Fig. 4). Hence, considering the data base as a whole (40 + 80% fractions) the generation of malondialdehyde exhibited highly significant negative correlations with the following movement characteristics after 24 h (Fig. 4): percentage of rapidly moving cells ($r = -0.588$; $P < 0.001$), percentage motility ($r = -0.664$; $P < 0.001$), mean path velocity ($r = -0.404$; $P < 0.01$), progressive velocity ($r = -0.410$; $P < 0.01$), curvilinear velocity ($r = -0.426$; $P < 0.01$), amplitude of lateral head displacement ($r = -0.340$; $P < 0.01$) and percentage progressive motility ($r = -0.631$; $P < 0.001$).

If the analysis was confined to the ability of the spermatozoa pelleting in 80% Percoll to sustain their motility over 24 h, then similar negative correlations were observed: percentage of rapidly moving cells ($r = -0.486$; $P < 0.01$), percentage motility ($r = -0.623$; $P < 0.001$), mean path velocity ($r = -0.421$; $P < 0.05$), progressive velocity ($r = -0.379$; $P < 0.05$), curvilinear velocity ($r = -0.464$; $P < 0.05$), amplitude of lateral head displacement ($r = -0.453$; $P < 0.05$) and percentage progressive motility ($r = -0.567$; $P < 0.01$).

Sperm–oocyte fusion

The generation of malondialdehyde was not only correlated with the capacity of human spermatozoa to exhibit motility but also with their ability to engage in sperm–oocyte fusion (Fig. 5). The levels of sperm–oocyte fusion observed with the 80% Percoll fractions isolated from this population of donors were significantly ($P < 0.001$) higher (87.7 ± 5.4% oocytes penetrated with a mean of 8.3 ± 1.2 spermatozoa fusing with each egg) than from the corresponding 40% fractions (29.7 ± 7.2% penetration with 0.9 ± 0.4 spermatozoa per egg). If the levels of oocyte fusion recorded for the entire data base were plotted against malondialdehyde generation in the presence of promoter, then a clear negative relationship between these elements was observed (Fig. 5). The low levels of sperm–oocyte fusion observed in the 40%, and occasional 80%, Percoll fractions were associated with high levels of malondialdehyde generation. There were also three samples in which low levels

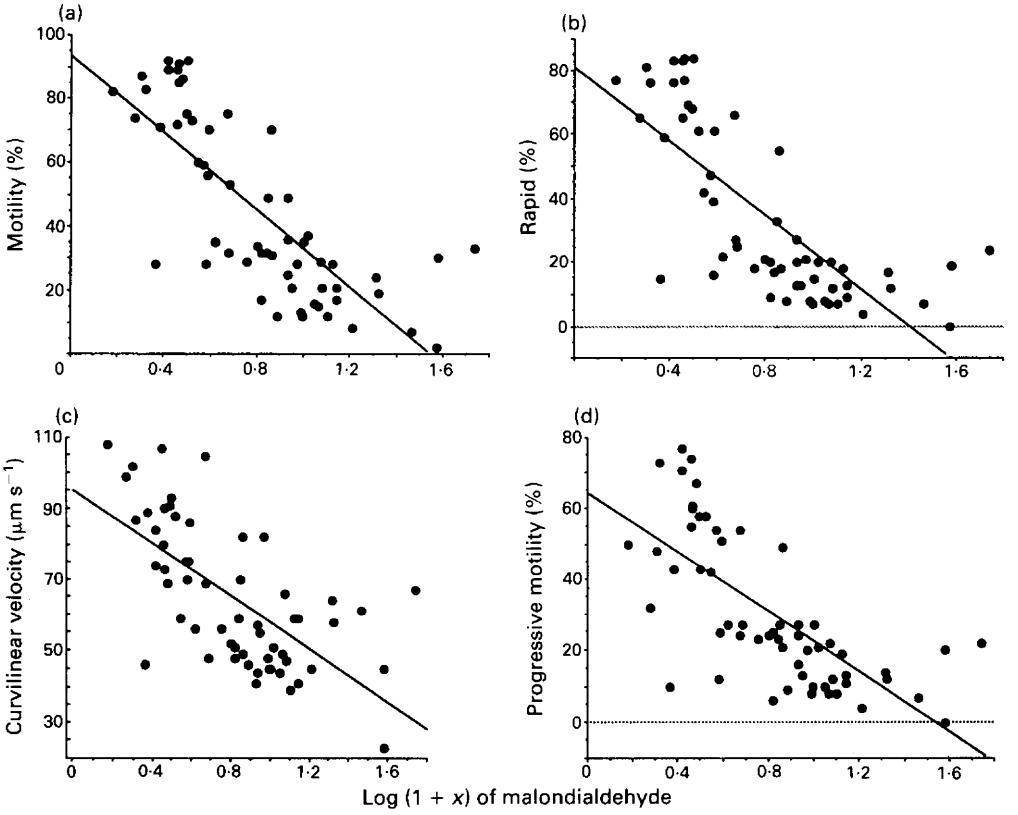

Fig. 2. Relationship between the generation of malondialdehyde and the movement characteristics of human spermatozoa after 3 h incubation *in vitro* for total data set. (a) Percentage motility, (b) percentage rapid, (c) curvilinear velocity and (d) percentage progressive motility.

of sperm–oocyte fusion were recorded in the presence of a low potential for malondialdehyde generation (Fig. 5). In such cases other factors related to the structure or motility (one of these samples exhibited $<10\%$ motility following incubation with A23187) of the spermatozoa must be responsible for the loss of sperm function. However, whenever high levels of malondialdehyde generation were observed, in either Percoll fraction, sperm–oocyte fusion was invariably impaired. As a result, significant negative correlation coefficients were observed between malondialdehyde generation and sperm–oocyte fusion for both the entire data set ($r = -0.58$; $P < 0.001$) and the 80% fraction ($r = -0.432$; $P < 0.05$).

Discussion

The generation of malondialdehyde in the presence of a ferrous ion promoter represents the catalytic breakdown of lipid hydroperoxides in the sperm plasma membrane and the subsequent propagation of a peroxidation chain reaction through the creation of alkoxyl and peroxyl radicals according to the following scheme (Gutteridge, 1984; Aitken *et al.*, in press):

$$\underset{\text{Lipid hydroperoxide}}{LOOH} + Fe^{2+} = \underset{\text{alkoxyl radical}}{LO^{\bullet}} + OH^{-} + Fe^{3+}$$

$$\underset{\text{Lipid hydroperoxide}}{LOOH} + Fe^{3+} = \underset{\text{peroxyl radical}}{LOO^{\bullet}} + H^{+} + Fe^{2+}$$

The iron promoted TBA assay therefore measures the two aspects of lipid peroxidation in spermatozoa: (i) the availability of lipid hydroperoxides in the sperm plasma membrane with which to initiate the peroxidative chain reaction and (ii) the ability of the spermatozoa to inhibit the propagation of this process, either through the action of chain-breaking antioxidants, such as α-tocopherol, or through the enzymatic cleavage and reduction of the lipid hydroperoxides (LOOH) to stable hydroxy acids (LOH). The latter could be achieved through the combined action of phospholipase A_2, to cleave out the peroxidized fatty acid, and glutathione peroxidase, to effect its reduction. Although both of these enzyme systems have been described in human spermatozoa (Bennet *et al.*, 1987; Alvarez and Storey, 1989), it is not known whether the species of glutathione peroxidase present in this cell type can reduce lipid hydroperoxides, as well as hydrogen peroxide. However, since glutathione peroxidase activity in human spermatozoa is relatively invariate (Alvarez *et al.*, 1987) and there is no evidence for vitamin E deficiency in infertile men, the most significant variable being monitored by the TBA assay is probably the availability of lipid hydroperoxides in the sperm plasma membrane with which to initiate the peroxidation chain reaction.

The existence of a good correlation between the generation of malondialdehyde and the ability of human sperm suspensions to produce reactive oxygen species (Aitken *et al.*, in press) is in keeping with this interpretation. The excessive production of

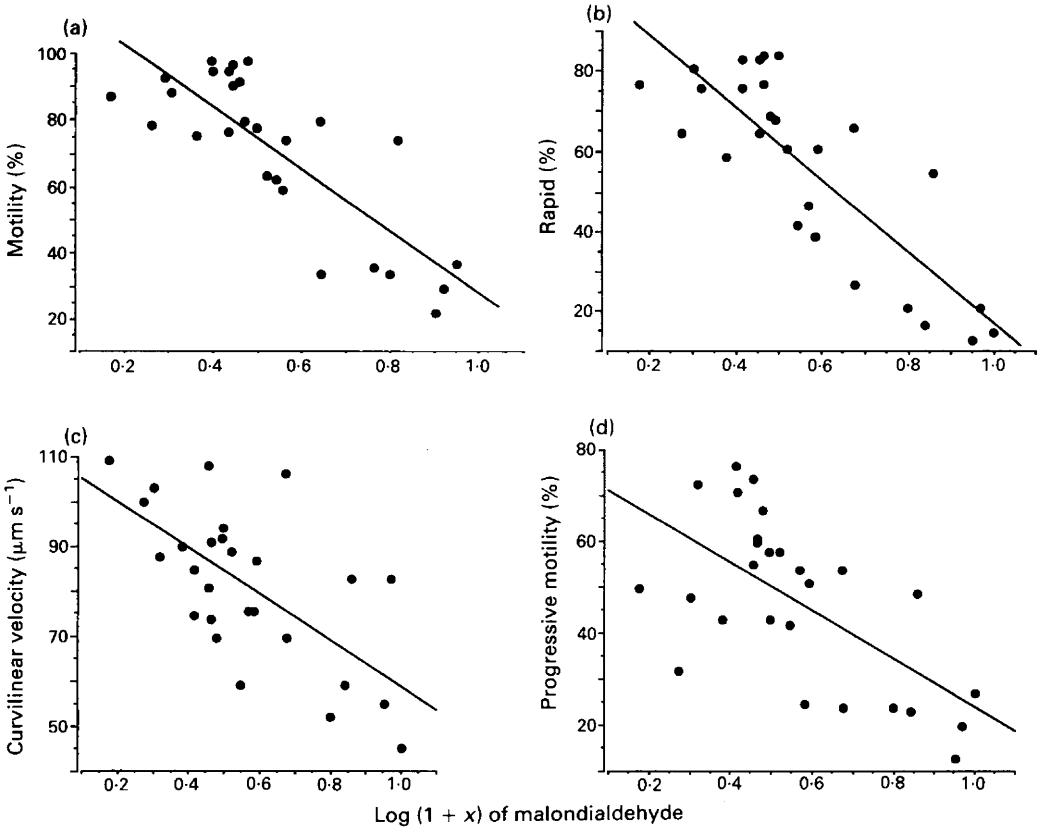

Fig. 3. Relationship between the generation of malondialdehyde by human spermatozoa pelleting in 80% Percoll and their movement characteristics after 3 h incubation *in vitro*. (a) Percentage motility, (b) percentage rapid, (c) curvilinear velocity and (d) percentage progressive motility.

Table 3. Movement characteristics of human spermatozoa after 24 h incubation

Criterion	40%–80% Percoll interface	80% Percoll
Percentage rapid	8.2 ± 1.1***	31.4 ± 4.8§***
Percentage motile	15.6 ± 1.7***	44.0 ± 5.4§***
Mean path velocity (μm s^{-1})	32.2 ± 2.1**	38.3 ± 3.1***
Mean progressive velocity (μm s^{-1})	26.0 ± 1.9**	32.9 ± 2.9***
Mean curvilinear velocity (μm s^{-1})	45.7 ± 2.8**	52.6 ± 4.3***
Beat frequency (Hz)	11.0 ± 0.7	10.9 ± 0.6*
Amplitude of head displacement (μm)	3.2 ± 0.3*	3.6 ± 0.3***
Progressive motility (%)	10.2 ± 1.2	31.6 ± 4.0§

§$P < 0.001$ (40% versus 80% Percoll fraction).
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (3 h versus 24 h motility).

superoxide and hydrogen peroxide either by defective spermatozoa (Aitken *et al.*, 1992b) or infiltrating leucocytes (Aitken and West, 1990; Kessopoulou *et al.*, 1992) would be expected to initiate peroxidation through Fenton or Haber Weiss reactions, in which hydroxyl radicals ($HO^{\bullet}$) would be formed through the catalytic action of extracellular transition elements such as iron or copper (Kwenang *et al.*, 1987). $HO^{\bullet}$ is an aggressive, highly reactive radical species, that will readily initiate the peroxidation of unsaturated fatty acids by means of a hydrogen subtraction process that leads to the formation of carbon-centred lipid radicals. Molecular stabilization of such lipid radicals creates conjugated dienes which combine with oxygen to generate peroxyl radicals that ultimately stabilize in the plasma membrane as lipid hydroperoxides. Further propagation of

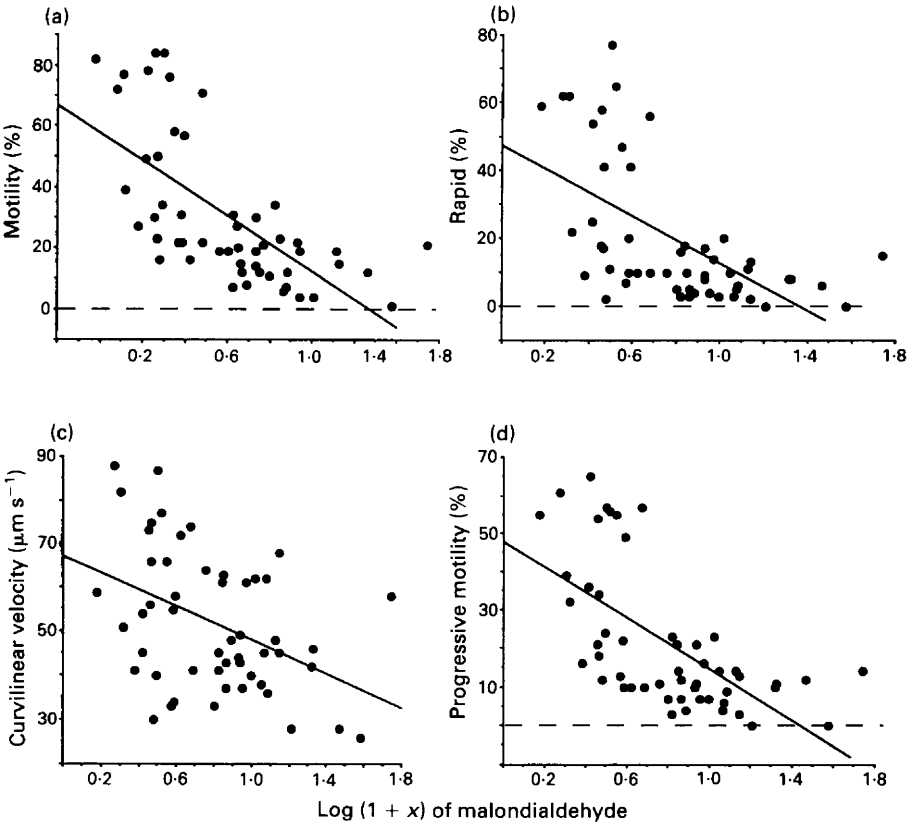

Fig. 4. Relationship between the generation of malondialdehyde and the movement characteristics of human spermatozoa after 24 h incubation *in vitro* for total data set. (a) Percentage motility, (b) percentage rapid, (c) curvilinear velocity and (d) percentage progressive motility.

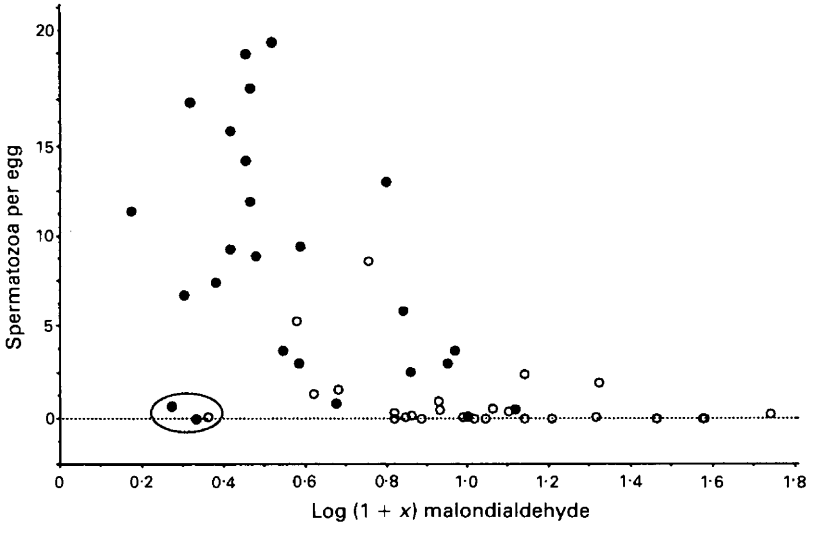

Fig. 5. Relationship between the generation of malondialdehyde and the capacity of human spermatozoa for sperm–oocyte fusion. (●) 80% Percoll fractions, (○) 40% Percoll fractions. Circle encloses three samples in which a failure to exhibit sperm–oocyte fusion was associated with low levels of malondialdehyde generation.

the lipid peroxidation chain reaction would be prevented by a combination of enzymatic (phospholipase A_2, glutathione peroxidase) and non-enzymatic (α-tocopherol, lactoferrin) mechanisms. As a consequence, spermatozoa that have suffered oxidative stress would be characterized by the accumulation of lipid hydroperoxides in their plasma membranes, which would

be relatively stable until induced to decompose and liberate malondialdehyde upon addition of the ferrous ion promoter used in the TBA assay.

The build-up of lipid hydroperoxides in the sperm plasma membrane clearly had a profound negative impact on sperm function. This applied to the variation in functional competence observed both within (the 40% versus the 80% Percoll fractions) and between ejaculates. In terms of the movement characteristics of human spermatozoa, lipid peroxidation influenced not just the percentage of motile spermatozoa, as reported originally by Jones *et al.* (1978, 1979), but also the pattern of sperm movement, reducing the average path, progressive and curvilinear velocities as well as the amplitude of lateral head displacement. Of particular interest was the relationship observed between the ability of human spermatozoa to generate malondialdehyde in the presence of promoter and their capacity for sustained motility over the next 24 h. The prolonged survival of human spermatozoa *in vitro* is of clinical importance in the practice of *in vitro* fertilization therapy (Cohen *et al.*, 1985) and as a quality control criterion (Critchlow *et al.*, 1989). In view of the apparent importance of lipid peroxidation potential in predicting the capacity of human spermatozoa to survive *in vitro*, the development of culture media supplemented with antioxidants to limit this kind of damage would seem rational.

The mechanism by which the accumulation of lipid hydroperoxides in the spermatozoon might inhibit motility has yet to be elucidated. In other cell types the membrane stabilizing effects of lipid peroxidation have been found to disrupt the activity of key membrane-bound enzymes, such as the ATPases (Ohta *et al.*, 1989). As a consequence of such damage, the regulation of intracellular cations such as calcium (Hirosumi *et al.*, 1988), which are known to play an important role in the control of human sperm motility (Hong *et al.*, 1984), would become disrupted. The inappropriate activation of calcium-dependent enzymes such as phospholipase A2, might then amplify the effects of such disruption by catalyzing the loss of fatty acids from the plasma membrane, as has been observed with hepatocytes following peroxidative damage (Ungemach, 1985).

The increase in membrane order that results from lipid peroxidation (Block, 1991) might also underly the relationship between peroxidative damage and the impaired ability of human spermatozoa to exhibit sperm–oocyte fusion in response to the second messengers generated by A23187. An association between the potential of human spermatozoa to generate malondialdehyde and an impaired capacity for sperm–oocyte fusion supports previous results indicating that the inability of human spermatozoa to fuse with zona-free hamster oocytes is correlated with the excessive generation of reactive oxygen species (Aitken and Clarkson, 1987a, b; Aitken *et al.*, 1989a, b, 1991). The resultant generation of hydroperoxides would be expected to lead to an increase in membrane rigidity, as a consequence of which the capacity of human spermatozoa to engage in events such as sperm–oocyte fusion, which depend on membrane fluidity, would be impaired.

Although the excessive generation of reactive oxygen species by defective spermatozoa is particularly common in cases of oligozoospermia (Aitken *et al.*, 1992b), it is important to note that only one of the patients analysed in this study exhibited this condition. However asthenozoospermia and teratozoospermia was evident within the study population and it may be the defects in morphology that hold the key to understanding the origins of the peroxidative damage recorded in this study. The spermatozoa isolated from the 40% and 80% Percoll fractions exhibited evidence of lipid peroxidation that was correlated with abnormalities of the sperm midpiece, but not the head or flagellum. Defects of the midpiece were defined in terms of deviation from the World Health Organization's (1987) definition of normality, the most common defect being the presence of excess cytoplasm. In the hierarchical scoring system used in this study the cells scored as having a defective midpiece would not have included those spermatozoa possessing an abnormal midpiece in association with defective head morphology. In this sense, the correlation between midpiece morphology and lipid peroxidation may have been understated and studies are in progress in which a more refined analysis of midpiece morphology is being conducted using image analysis techniques. It is significant that a previous study (Rao *et al.*, 1989) also found that the lipid peroxidation potential of human spermatozoa might be linked to the incidence of mid-piece defects. The significance of such a relationship may lie in the important role that substrate availability plays in the generation of reactive oxygen species in human spermatozoa. The NADPH oxidase that is responsible for this activity (Aitken and Clarkson, 1987a, b) derives its substrate (NADPH) from a biochemical pathway, the hexose monophosphate shunt, that is located in the cytoplasm of the cell (C. Ford and J. Aitken, unpublished observations). Spermatozoa with abnormally large midpieces would be expected to generate unusually high amounts of NADPH, as a consequence of which reactive oxygen species generation, and thence lipid peroxidation, would be enhanced. The existence of causal relationships between midpiece morphology, cytoplasmic volume, NADPH availability, reactive oxygen species generation and lipid peroxidation, might explain why a number of recent studies have demonstrated an association between male infertility and the cellular content of another cytoplasmic enzyme, creatine phosphokinase (Huszar *et al.*, 1988a, b, 1990).

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