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Thiols of flagellar proteins are essential for progressive motility in human spermatozoa

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Abstract. Male infertility is a disorder of the reproductive system defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse. The presence of low-motile or immotile spermatozoa is one of many causes of infertility; however, this observation provides little or no information regarding the pathogenesis of the malfunction. Good sperm motility depends on correct assembly of the sperm tail in the testis and efficient maturation during epididymal transit. Thiols of flagellar proteins, such as outer dense fibre protein 1 (ODF1), are oxidised to form disulfides during epididymal transit and the spermatozoa become motile. This study was designed to determine how oxidative changes in protein thiol status affect progressive motility in human spermatozoa. Monobromobimane (mBBr) was used as a specific thiol marker and disruptor of sperm progressive motility. When mBBr was blocked by dithiothreitol it did not promote motility changes. The analysis of mBBr-treated spermatozoa revealed a reduction of progressive motility and an increased number of spermatozoa with non-progressive motility without affecting ATP production. Laser confocal microscopy and western blot analysis showed that one of the mBBr-positive proteins reacted with an antibody to ODF1. Monobromobimane fluorescence intensity of the sperm tail was lower in normozoospermic than asthenozoospermic men, suggesting that thiol oxidation in spermatozoa of asthenozoospermic men is incomplete. Our findings indicate that mBBr affects the thiol status of ODF1 in human spermatozoa and interferes with progressive motility.

Additional keywords: cytoskeleton, epididymus, fertility.

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Introduction

Good sperm motility is an essential factor in normal male fertility. Men with low-motile or immotile spermatozoa are typically infertile or sterile. However, assigning the classification 'subfertile' or 'infertile' in a clinical setting on the basis of low sperm motility provides little or no information regarding the pathogenesis of the particular case (Turner 2006).

Good motility depends on correct assembly of the sperm tail in the testis in combination with efficient maturation during epididymal transit (Cooper 2011). The sperm tail contains the axoneme, which is highly conserved in all ciliated and flagellated eukaryotic cells, and certain accessory structures found only in mammalian sperm flagella (Turner 2006), in particular the mitochondrial sheath, outer dense fibres (ODFs) and fibrous sheath. These structures are assembled during spermatogenesis through a precise arrangement of microtubules, mitochondrial migration and Golgi transformation (Kierszenbaum and Tres 2004; Xiao and Yang 2007).

Proteins rich in thiols have been found in the nucleus, ODFs, connecting piece, fibrous sheath and outer mitochondrial membrane (Vera *et al.* 1984; Oko 1988; Seligman *et al.* 1994). ODFs are a component of the prominent cytoskeleton of the sperm cell (Olson and Sammons 1980; Oko 1988). Previous studies have suggested that the function of ODFs is related to the bending torque of the tail (Lindemann 1996) because of the elastic structure of the sperm tail (Baltz *et al.* 1990). ODFs may play

a protective role against shear forces during epididymal transport and ejaculation (Baltz *et al.* 1990). They may also support axonemal beating through force transmission to the flagellar base (Lindemann 1996). Impaired development of the ODFs has been described as a major cause of tail abnormalities in infertile men (Haidl *et al.* 1991).

Some infertility cases with a high number of decapitated seminal spermatozoa may involve loss of ODF function (Haidl *et al.* 1991). Sperm motility is reduced in heterozygous mice with impairment of the ODF1 N-terminal fragment, suggesting a similar cause for certain cases of reduced fertility in humans (Yang *et al.* 2012). On the other hand, changes in repeat C-X-P frequencies in ODF1 do not affect fertility in humans (Hofferbert *et al.* 1993).

During epididymal transit, sperm proteins undergo sulfhydryl oxidation and spermatozoa concurrently acquire progressive motility (Cornwall *et al.* 1988). Changes of flagellar beat and motility pattern in spermatozoa (Bedford *et al.* 1973) are associated with a shift from flexible to more rigid flagella during epididymal transit (Cornwall and Chang 1990).

ODF1 (Cabrillana *et al.* 2011; Dias *et al.* 2014) and other ODF proteins (Cornwall and Chang 1990) undergo maturational changes (thiol oxidation) during epididymal transit in various mammalian species. These changes involve a reduction in the availability of thiol groups and of solubility in sodium dodecyl sulfate (SDS). We demonstrated that the thiol-reactive fluorescent probe monobromobimane (mBBr) reduces rat sperm motility in a dose- and time-dependent manner (Cabrillana *et al.* 2011).

Fluorescent labelling with bimanes provides a convenient, sensitive method for assessing protein thiol status under physiological conditions. Because of its lipophilic character (Kosower *et al.* 1979; Kosower and Kosower 1987) mBBr readily penetrates intact living cells and selectively modifies free cysteine (Cys) residues in proteins under physiological conditions. The reaction of bimanes with sulfhydryl groups of free Cys results in a highly fluorescent derivative (Petrotchenko *et al.* 2006). We demonstrated by matrix-assisted laser desorption/ionization (MALDI) mass spectrometer (MS) that mBBr labels Cys in the N-terminal domain of ODF1 (Cabrillana *et al.* 2011).

In the present study, we tested the hypothesis that changes in thiol status of flagellar proteins affect progressive motility in human spermatozoa, using mBBr as a specific thiol marker and sperm motility disruptor without affecting sperm vitality and ATP production. ODF1 was characterised as a mBBr-positive protein in human spermatozoa. Samples of asthenozoospermic men were used as a cellular model that lost sperm progressive motility without chemical treatment. The thiol status of spermatozoa from asthenozoospermic men was compared with that of normozoospermic men to clarify the relationship between thiol status and progressive motility.

Materials and methods

Chemicals

Ham's F-10 medium was from Laboratorio Microvet. Monobromobimane and DTT were from Sigma Aldrich. All other reagents (analytical grade) were from local companies as Laboratorio Biofarma S.A. indicated. Monobromobimane stock solution was prepared at 150 mM in acetonitrile and stored in aliquots at −18°C.

Sample collection

Semen samples were collected between early 2011 through to late 2012 at the Institute of Reproductive Medicine (Mendoza, Argentina). The study protocol was approved by the Ethics Committee of the local School of Medicine (Facultad de Ciencias Médicas, Universidad Nacional de Cuyo, resolution #7982/2010, http://fcm.uncuyo.edu.ar/bioetica; verified 8 June 2016). Male patients were informed of the purpose of the study. Volunteer subjects ($n = 46$; age range 27–38 years) were divided into a control group (normozoospermic men, $n = 31$) and an asthenozoospermic group (patients with idiopathic asthenozoospermia, $n = 15$). Each subject provided a single semen sample.

Semen analysis

Semen samples were obtained by masturbation (following ≥3 days without ejaculation), allowed to liquefy for 30 min and analysed promptly according to WHO criteria (World Health Organisation 2010). The results were expressed as median (5th–95th percentile). The control group presented the following characteristics: 70 (34–147.6) × 10^6 spermatozoa mL^{-1}, progressive motility 53 (31–73.4) %, live cells 83 (67–95.6) %, normal sperm morphology 15 (10–40.5) % and round cell plus leukocytes $<1 \times 10^6$ cells mL^{-1}. On the other hand, the asthenozoospermic group had a sperm concentration of 31 (2–110) × 10^6 spermatozoa mL^{-1}, progressive motility 28.5 (25–31) %, live cells 75 (65–92) %, normal sperm morphology 7 (4–16) % (strict criteria, Menkveld *et al.* 1990) and round cell and leukocytes $<1 \times 10^6$ cells mL^{-1}.

Motile sperm isolation technique

Motile spermatozoa were isolated by the direct swim-up technique of Mortimer (1994). In brief, 1 mL of liquefied semen was layered under 1 mL Ham's F-10 without albumin. The tube was oriented at ~45° and kept in an incubator (Napco) under 5% CO_2 atmosphere for 1 h at 37°C. The tube was slowly returned to an upright position, the upper 500 µL of medium was removed and sperm concentration was adjusted to 1×10^7 cells mL^{-1} with fresh medium.

Effects of mBBr on sperm motility

We have previously demonstrated that mBBr inhibits sperm motility in rats (Cabrillana *et al.* 2011). The effect of mBBr on human sperm motility has not been studied previously.

Three samples of normozoospermic men were combined with 0.3 mM mBBr and sperm motility was assessed during a 60-min period. Three samples of asthenozoospermic men were processed similarly, except that the assessment period was 30 min because sperm motility decreased quickly.

To determine the minimal concentration of mBBr that altered sperm motility, aliquots of sperm suspension of normozoospermic men were incubated for 60 min at 37°C under 5% CO_2 with

Table 1. Effects of mBBr on sperm progressive motility

The percentage of progressive sperm motility was measured every 10 min during 1 h in samples without mBBr (Control) or treated with various concentrations of mBBr as shown. Means for the experimental conditions were compared with control value at time zero (means ± s.e.m.); $*P < 0.01$, $***P < 0.001$

Time (min)	Sperm progressive motility (%)				
	Control	mBBr (mM)			
		0.003	0.03	0.3	3
0	83.66 ± 9.81	70.33 ± 15.04	67 ± 12.76	64.43 ± 6.5	64.33 ± 13.5
10	78.33 ± 11.50	66 ± 16.97	69 ± 12.72	28 ± 2.64*	14.3 ± 4.04*
20	78 ± 12.52	66.5 ± 16.2	66 ± 16.97	25 ± 3.46*	4 ± 1*
30	78 ± 16.29	68.5 ± 12.02	61 ± 9.19	11.66 ± 2.08*	0 ± 0*
60	73 ± 22.5	29.5 ± 20.50	30 ± 28.28	0 ± 0***	0 ± 0***

Ham's F-10 alone (control) or Ham's F-10 plus various concentrations of mBBr (0.003, 0.03, 0.3 and 3 mM; Table 1).

DTT blocks the effect of mBBr on sperm progressive motility

Possible effects on sperm motility by reactive groups present on mBBr were also investigated. Monobromobimane forms a stable and irreversible thioether linkage with free thiol groups of Cys residues (Kosower *et al.* 1979; Kosower and Kosower 1987). Another control was performed by pre-blocking of mBBr reactive groups by DTT to prevent sulfhydryl (SH)-related inhibition of sperm motility. Dithiothreitol (3 mM) was co-incubated (1 : 1) with 0.3 mM mBBr for 1 h at room temperature (RT) in the dark to ensure reaction. Aliquots of spermatozoa from normozoospermic and asthenozoospermic men were added with Ham's F-10 plus blocked mBBr. The effect of DTT on motility was assessed using Ham's F-10 plus 3 mM DTT. Finally the effect of acetonitrile on sperm motility was checked (5 µL of acetonitrile was added to 250 µL of sperm suspension, without mBBr).

At 10-min intervals, a 20-µL sample from each of the above conditions was placed on a warmed slide (37°C), covered with a 22-mm coverslip and examined under a microscope. Progressive sperm motility was determined by counting at random the number of progressively motile spermatozoa in a population of 200 cells. For each condition, the percentage of viable cells (mean ± s.e.m.) was calculated.

Analysis of sperm vitality

Sperm vitality was determined by the hiposmotic (HOS) test. In brief a 5-µL sperm sample was mixed with 45 µL hypo-osmotic medium (0.735 g sodium citrate dihydrate and 1.351 g D-fructose in 100 mL of purified water; 150 mOsm kg^{-1} confirmed by a Wescor 5500 cryoscopic vapour pressure osmometer) and incubated for 5 min at 37°C. A 20-µL aliquot of the mixture was placed on a warmed slide, coverslipped and spermatozoa (>200) were examined under a microscope. The numbers of spermatozoa with a curled tail (viable) and non-curled tail (non-viable; Jeyendran *et al.* 1984) were recorded. For each condition, the percentage of viable cells (mean ± s.e.m.) was calculated.

Analysis of sperm movement

Our previous study showed that mBBr reacts with Cys thiols and reduces rat sperm motility in a dose- and time-dependent manner (Cabrillana *et al.* 2011). Here we examined the possibility that thiol groups are similarly involved in motility of human sperm cells.

Sperm motility in the presence of mBBr was assessed in a Makler counting chamber (Sefi-Medical Instruments) using three samples of normozoospermic men. Movements of control and mBBr-treated cells were recorded with a Hamamatsu C4742–95 camera (Hamamatsu Photonics) connected to a PC (Dell Argentina) with the MetaMorph software program (Molecular Devices), attached to an inverted microscope (Nikon Eclipse T2000; Nikon) in a constant-temperature chamber at ~37°C.

Motility was monitored for 1 min at 5-min intervals during a total period of 30 min. The images were compiled using the MetaMorph program to produce a video (1 min; 800 frames) of sperm motility. Sperm tracking in the video was analysed using Image J software (Version 1.46; NIH) with the ParticleTracker3Dmodular_ java 210 plugins. This is a well-established program that provides reliable data (Image J 1.46j; https://imagej.nih.gov/ij/download.html; Meijering *et al.* 2012).

Trajectories of individual sperm cells were projected onto *x* and *y* axes by the program, with *x*/*y* values expressed in pixels. Data were converted to µm using a Visual Basic macro that was designed by Matias Fornés. The program uses a mathematical technique (following section) to calculate values of straight-line displacement (generally called SL), curvilinear path (CP or total trajectory) and curvilinear velocity (VCL) of a sperm population. For each condition, trajectories of 300 cells were measured in three separate experiments and results were expressed as mean ± s.e.m. This approach provides a detailed analysis of sperm movement.

Mathematical technique for analysis of sperm progressive motility

The distance between grid lines in the Makler chamber is 100 µm, so there is a conversion factor of 0.7 µm per pixel when using the 25× microscope objective. SL, CP and VCL were calculated using the following equations:

Equation 1: straight-line displacement (SL) is the vector distance from point A (start) to point B (end) described by a spermatozoon.

Equation 2: curvilinear path or total trajectory (CP) is the total distance travelled by a spermatozoon along a curved path from point A (start) to point B (end).

Equation 3: curvilinear velocity (VCL) is the rate of change of position (curvilinear path) during 1 min.

$$\Delta x = (x_{end} - x_{start}) \times \text{scale}$$
$$\Delta y = (y_{end} - y_{start}) \times \text{scale} \quad (1)$$

$$X = \sum \Delta x_i \times ...\text{scale}$$
$$Y = \sum \Delta y_i \times ...\text{scale} \quad (2)$$

$$V_i = \frac{\sqrt{\Delta x_i^2 + \Delta y_i^2}}{t} \quad (3)$$

Colocalisation of mBBr staining with ODF1

Aliquots (1 mL) of semen from three normozoospermic men were centrifuged at 500*g* for 15 min at room temperature and sperm cells were resuspended in culture medium at a final concentration 1×10^7 cells mL^{-1}. Two aliquots were obtained and 0.2 µL mBBr stock solution was added to one of them (final concentration 0.3 mM) and incubated for 20 min. Both aliquots were washed twice by centrifugation, resuspended in phosphate-buffered saline (PBS; 0.01 M phosphate buffer, 0.0027 M KCl, 0.137 M NaCl, pH 7.4), fixed with 2% (w/v) paraformaldehyde in PBS for 20 min, washed twice with PBS, incubated for 1 h in 0.1% (v/v) Triton X-100 and 5% (w/v) bovine serum albumin (BSA) in PBS, incubated with primary antibody anti-ODF1 (sc-27907, 1 : 200; Santa Cruz Biotechnology) for 1 h at RT, washed extensively with PBS and secondary antibody added (B7014, 1 : 500; Sigma Aldrich) for 15 min. Then cells were incubated with fluorescein (FITC)–streptavidin (Vector Laboratories Inc.) for 15 min. Cells were mounted with anti-fade Fluoroshield (Sigma Aldrich). Pictures were taken by confocal microscopy (model FV10-ASW; Olympus). Other aliquots with mBBr were processed as above but primary antibody was omitted.

Monobromobimane fluorescence intensity of sperm tail from normozoospermic and asthenozoospermic men

Differences in signal intensity generated by mBBr were only observed in the head of mature versus immature rat spermatozoa measured by flow cytometry (Shalgi *et al.* 1989). We previously reported differences in fluorescence signals in head and tail of mature and immature rat spermatozoa by measuring the fluorescence signal by microscope (Cabrillana *et al.* 2011).

For the present experiments, 1 mL semen from normo- and asthenozoospermic men was centrifuged al 500*g* for 15 min at room temperature. For each of the two types of samples, two aliquots were obtained and one was incubated with 30 mM DTT (to reduce disulfide bonding to sulfhydryl groups; Cleland 1964) for 20 min at 37°C. Pellets from normozoospermic, normozoospermic+DTT, asthenozoospermic and asthenozoospermic+DTT aliquots were washed twice with PBS and resuspended in culture medium to a final concentration of 1×10^7 cell mL^{-1}. Monobromobimane (0.2 µL stock solution) was added to 100 µL of each sperm suspension (final concentration 0.3 mM) and incubated for 20 min (Kosower *et al.* 1979; Kosower and Kosower 1987; Shalgi *et al.* 1989). For each experimental condition, fluorescence intensity of 200 sperm cells was estimated using the Image J program ('Analyze' option, histogram function applied to a limited area (middle and principal piece) of sperm tail). This measurement was termed relative fluorescence intensity (RFI). RFI was calculated for each condition in three separate experiments and mean, minimum and maximum values were recorded.

Identification of mBBr-labelled proteins and western blot analysis for ODF1

Two semen samples of normo- and asthenozoospermic men were washed twice with 1× PBS and incubated with 0.3 mM mBBr (final concentration) for 20 min. After that the samples were washed and the pellet was subjected to osmotic shock by 5 min incubation in 0.5× Ham's F-10 and then incubated 10 min with 10 mM ethylenediamine tetraacetic acid (EDTA) and 10 mM DTT. Each sample was sonicated at 10 W in an ultrasonicator (Kontes) for five cycles of 30 s at RT. Osmolarity was adjusted by addition of 10× PBS, then samples were combined with Laemmli sample buffer (0.0625 M Trizma Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 0.01% (w/v) bromophenol blue) and DTT concentration was adjusted to 20 mM (Lishko *et al.* 2010). Samples were sonicated for another 5 min and boiled for 5 min. Total cell lysates and the molecular-weight standard (wide-range, dual colour; Bio-Rad) were loaded onto a 12.5% (w/v) acrylamide gel with 4% (w/v) stacking gel (Laemmli 1970). Monobromobimane fluorescence intensity observed in the acrylamide gel was recorded by a camera (model LAS 4.000; Fujifilm). Proteins were transferred to a nitrocellulose membrane (Towbin *et al.* 1979). For immunoblotting, the membrane was incubated overnight in blocking buffer (3% (v/v) *Teleostean* gelatin fish in Towbin buffer (TBS)-T, 1.92 mM Tris base, 0.1% (v/v) Tween 20) at 4°C, incubated 1 h at RT with primary antibody anti-ODF1 (1 : 5000), washed twice with TBS-T and incubated with biotin-conjugated anti-goat IgG antibody (B7014, 1 : 3000; Sigma Aldrich) for 1 h at RT. Bound antibody was visualised by enhanced chemiluminescence (1 M Tris base pH 8.5, 250 mM luminol, 90 mM cumaric acid, 3% (v/v) H_2O_2). The membrane was stained with Amido Black (Harper and Speicher 2001) and a chemiluminescence image (ODF1 signal) and Amido Black colour image (membrane staining proteins) were captured by the Fujifilm camera as above. Two western blots of anti-ODF1 and the fluorescent signal of mBBr obtained from two acrylamide gels were normalised by densitometry analysis using the Image J program ('Analyze' option, gel function applied, area under curve measured; Zarelli *et al.* 2009). The normalisation was expressed as the ratio between the mBBr signal versus Amido Black optical density and ODF1 blot versus Amido Black optical density. Molecular weight was calculated by the method of Ferguson (1964).

Effect of mBBr on ATP production

To assess the effect of mBBr on ATP levels motile spermatozoa selected by swim up from three normozoospermic samples were

used. The sperm suspension was separated into five aliquots. These were washed twice and incubated for 20 min at 37°C with either: (1) Ham's F10, (2) Ham's F10 without glucose but with 2.52 mM of 2-desoxi-D-glucose (DOG; glucose was replaced by an equal concentration of DOG, a competitive inhibitor of glucose to block glycolysis (Glyc); Mukai and Okuno 2004) and 40 μM rotenone (this reagent was added to inhibit oxidative phosphorylation (OxPhos); Nascimento *et al.* 2008), (3) Ham's F10 plus 0.3 mM mBBr co-incubated with 3 mM DTT, (4) Ham's F10 plus 0.3 mM mBBr or (5) Ham's F10 plus 30 mM DTT. Two aliquots from each of the five conditions detailed above, with 5×10^6 spermatozoa mL^{-1} of medium, were used to determine ATP level.

ATP production was assessed using the ENLITEN ATP assay kit (Promega Corporation), a luminescence method with the enzyme luciferase, which catalyses the formation of light from ATP, luciferin and oxygen. The generated luminescence intensity is proportional to the amount of ATP in the sample. For this, 50 μL of sperm suspension at 5×10^6 cells mL^{-1} was placed into a white-walled 96-well luminometer plate. Then, 5 μL of 0.5% of trichloroacetic acid (TCA, final concentration) was added as ATP-extracting reagent. Immediately the pH of the suspension was neutralised with 15 μL of Tris-acetate buffer (1 M Trisma, pH was adjusted to 7.7 with glacial acetic acid). Finally 100 μL of ATP reagent was added and incubated for 60 s at 25°C. The relative light units were measured with a luminometer (Fluoroskan; Ascent FL Thermo Scientific) and then converted to concentration values using a calibration factor of the standard solution and expressed as mM (mmol L^{-1}).

Statistical analysis

Data were analysed using the statistical packages GraphPad Prism 4 software (http://www.graphpad.com/scientific-software/prism; verified 8 June 2016). Unless noted otherwise, results presented in the text and figures are mean ± s.e.m. from three separate experiments performed in duplicate. Data for mBBr effect on sperm motility were analysed by two-way analysis of variance (ANOVA) for comparison of means with the control value. The Bonferroni post hoc test was used for all comparisons. Data were analysed by one-way ANOVA (Kruskal–Wallis test) and Dunn's post hoc test was used for all comparisons, to compare means between each time and initial time. RFI values and ratios of optical density measurements were analysed by the Kruskal–Wallis test and Dunn's post hoc test was used for all comparisons. Differences between means were considered to be significant when $P < 0.05$. All the data were analysed by Kolmogorov–Smirnov normality test before application of ANOVA and data found to be non-normal were subjected to non-parametric test.

Results

Effects of mBBr on sperm motility and vitality

A significant decline in the percentage of progressively motile spermatozoa in samples of normozoospermic men began after 10 min of incubation and was completed by 30 min for the group treated with 3 mM mBBr and 60 min for the group treated with 0.3 mM mBBr (two-way ANOVA results for comparison with control at time zero: $t = 3.818$; $P < 0.01$ and $t = 5.25$; $P < 0.001$ respectively; Table 1). For the 3 mM mBBr group only, the percentage of live spermatozoa was notably reduced at 60 min (HOS test, two-way ANOVA: $t = 7187$; $P < 0.001$; Fig. 1*b*). These findings indicate that mBBr primarily affects motility but not sperm vitality.

DTT blocks the effect of mBBr

The reactive group of mBBr was blocked by DTT to determine whether this group was responsible for the effect of mBBr on sperm progressive motility. The reduction in progressive motility was abrogated or blunted when mBBr was incubated with DTT before adding it to spermatozoa. When spermatozoa were incubated with DTT only (3 mM) a decrease in progressive motility was observed, but the difference was not significant in comparison with control (two-way ANOVA: $t = 0.8737$; $P > 0.05$; Fig. 1*a*). The vehicle had no significant effect on the sperm motility during the time frame of the experiment. The number of non-viable spermatozoa was not increased by any of these treatments (Fig. 1*b*).

Effect of mBBr on samples of asthenozoospermic men

When spermatozoa of asthenozoospermic men were incubated with 0.3 mM mBBr, a reduction in the percentage of motile spermatozoa was observed at 10 min, but the difference in comparison with control was not significant (two-way ANOVA: $t = 0.8838$; $P > 0.05$; Fig. 1*c*).

Treatments with vehicle, DTT or mBBr+DTT also had no significant effect on sperm motility or sperm vitality (Fig. 1*d*).

Effects of mBBr on sperm progressive motility: mBBr affects SLP and VCL but not CP

In the 0.3 mM mBBr group, a reduction in progressive sperm motility and corresponding increase in non-progressive spermatozoa were observed at 10 min. Video tracking revealed no difference in the percentage of immotile spermatozoa (Fig. 2*a*; see Figs S1 and S2, available as Supplementary Material to this paper). During incubation with mBBr, some of the motile spermatozoa began moving in the same place, but there was no change in the number of hyperactivated spermatozoa. This motility change may be related to the significant decreases observed in VCL (Kruskal–Wallis: $P < 0.001$; Fig. 2*b*) and in SL (Kruskal–Wallis: $P < 0.001$; Fig. 2*c*). No change was observed in CP (Kruskal–Wallis: $P = 0.6791$; Fig. 2*d*). The mean of each measurement obtained from sperm records (Fig. 2) was compared with value at time zero. A morphology evaluation was performed after record analysis.

Colocalisation of mBBr staining with ODF1

Samples of normozoospermic men were subjected to mBBr staining and immunodetection of ODF1. Sperm nuclei and tails were labelled by mBBr (Fig. 3*b1*, *c1*). The sperm tail, neck and perinuclear zone showed positive immunoreactivity with FITC-labelled anti-ODF1 (Fig. 3*a2*, *c2*). Colocalisation of mBBr with

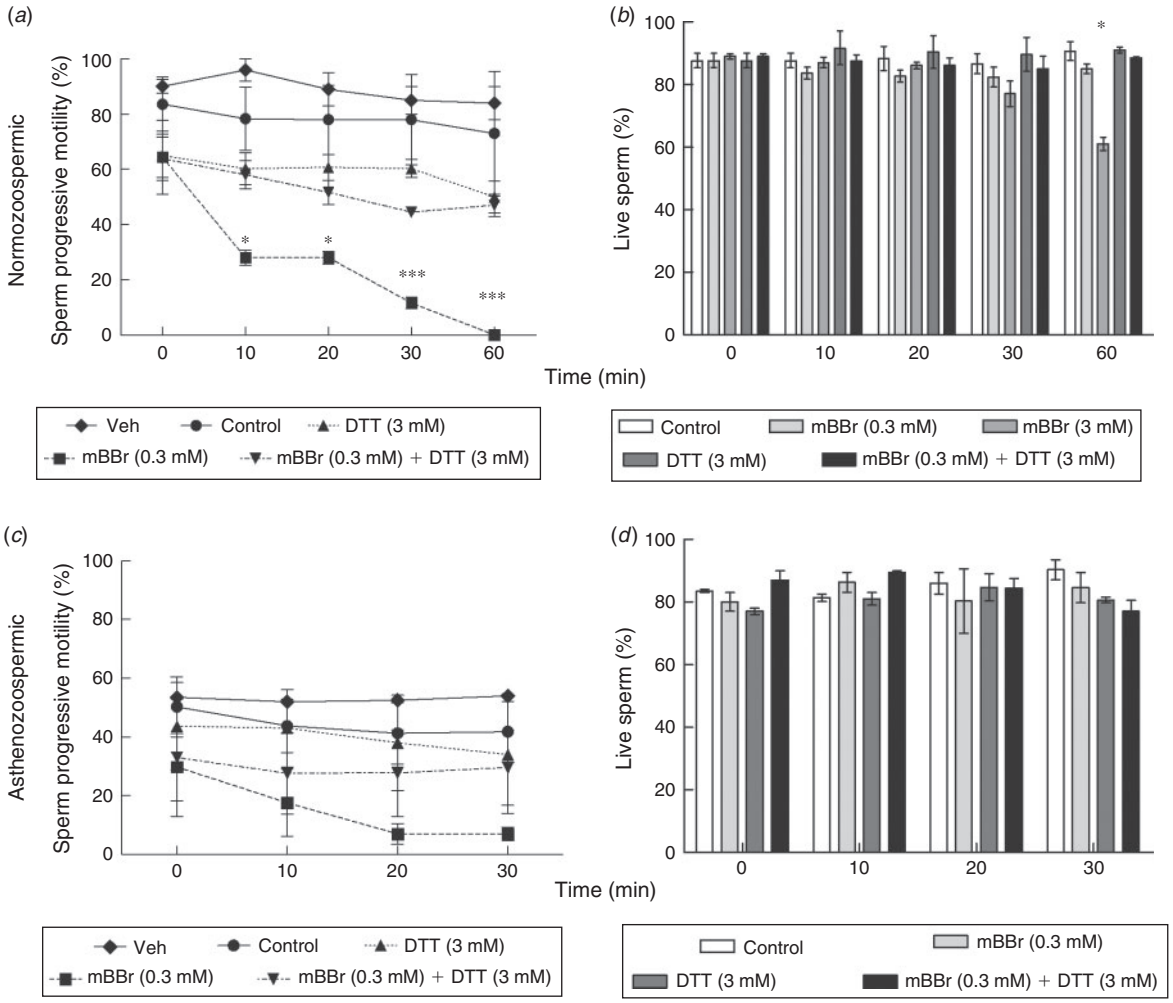

Fig. 1. Effects of mBBr on sperm motility and vitality. (*a*, *c*) Progressive sperm motility was expressed as a percentage of 200 spermatozoa counted. Sperm suspensions were placed in four tubes containing either acetonitrile (Veh), Ham's F-10 (Control) or various combinations of mBBr and DTT as indicated in the figure and examined at 0, 10, 20, 30 and 60 min. Each point represents the percentage (mean ± s.e.m.) of three separate experiments for samples from (*a*) normozoospermic and (*c*) asthenozoospermic men. (*b*, *d*) Sperm vitality was measured under the same experimental conditions described above. Bars represent the percentage (mean ± s.e.m.) of live spermatozoa for various treatments in samples from (*b*) normozoospermic and (*d*) asthenozoospermic men. Two-way ANOVA was performed to compare data points with control value at time zero. $*P < 0.01$, $***P < 0.001$.

ODF1 was observed in the sperm tail (Fig. 3*c4*, white arrow), neck and perinuclear zone (Fig. 3*c4*, white arrowhead).

Monobromobimane fluorescence intensity of sperm tail from normozoospermic and asthenozoospermic men

Monobromobimane-stained sperm tails from samples of normozoospermic men (Fig. 4*a*) showed lower fluorescence intensity than those from samples of asthenozoospermic men (Fig. 4*b*). The highest fluorescence intensity was observed for spermatozoa of normozoospermic men treated with 30 mM DTT and then stained with mBBr (Fig. 4*e*). For spermatozoa from asthenozoospermic men, DTT treatment had no effect on fluorescence intensity (Fig. 4*g*). Similar results were obtained for comparisons of RFI values of spermatozoa from normozoospermic and asthenozoospermic men (Kruskal–Wallis: $P < 0.001$; Fig. 4*i*).

Identification of mBBr-labelled proteins and western blot analysis for ODF1

Western blotting with anti-ODF1 antibody revealed a 14.8-kDa protein band coinciding with the fluorescent mBBr band in SDS-polyacrylamide gel electrophoresis (PAGE). Monobromobimane fluorescence intensity for this ODF1-positive band was lower in samples of normozoospermic men than in samples of asthenozoospermic men (Fig. 5*a*, number 4), but no difference was seen comparing ratios of gel fluorescence and ODF1 blot densitometry (Kruskal–Wallis: $P > 0.05$; Fig. 5*b*).

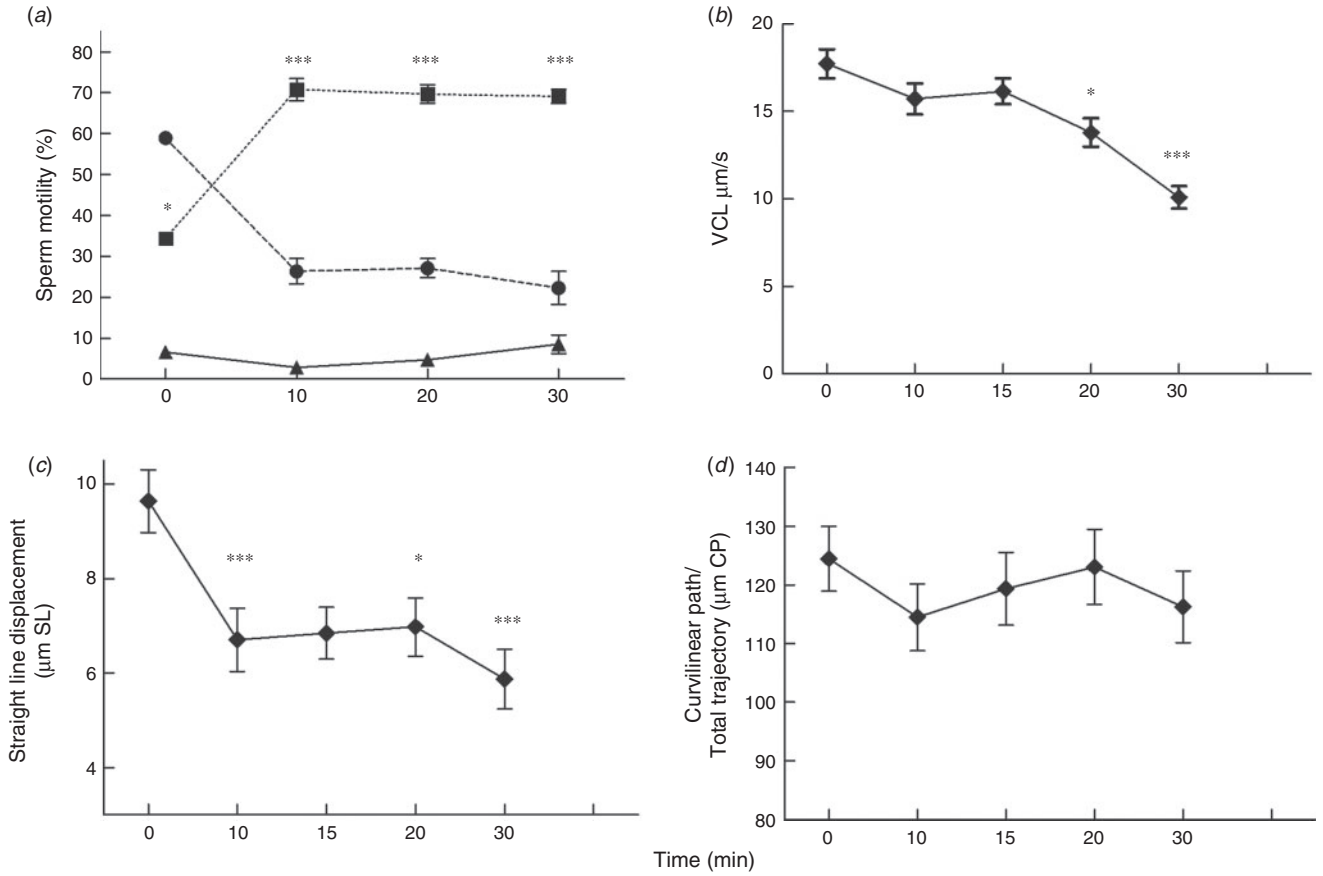

Fig. 2. Monobromobimane effects on sperm progressive motility; mBBr affects SL and VCL, but not CP. Effects of 0.3 mM mBBr on sperm motility of normozoospermic men were evaluated by calculating (*a*) percentages of progressive (●), non-progressive (■) and immotile (▲) spermatozoa and the parameters (*b*) curvilinear velocity (VCL), (*c*) straight-line displacement (SL) and (*d*) curvilinear path or total trajectory (CP). Sperm tracking data were analysed and expressed as mean ± s.e.m. Means of measurements obtained from sperm records were compared between various time points and value at time zero. $*P < 0.01$, $***P < 0.001$.

Effect of mBBr on ATP production

When spermatozoa were incubated with DOG and rotenone (−Glyc −OxPhos CONTROL), a reduction in ATP level was observed as expected (one-way ANOVA: $t = 7.319$, $P < 0.001$; Fig. 6). In contrast, no reduction in ATP production was noted when the spermatozoa were incubated with mBBr, mBBr blocked with DTT or DTT alone in comparison with the control (one-way ANOVA: $t = 1.39$, $t = 2.259$, $t = 6.651$ respectively, $P > 0.05$; Fig. 6).

Discussion

This study was designed to evaluate the contribution of flagellar proteins rich in thiols to sperm motility. When Cys residues were blocked by mBBr, progressive motility was reduced without affecting ATP production or sperm vitality. When mBBr was blocked by DTT, the effect on sperm motility was avoided.

The mBBr fluorescence signal in sperm tail was lower for normozoospermic than for asthenozoospermic men. This difference could indicate that an adequate amount of thiols in flagellar proteins is necessary for good motility. Western blotting analysis and confocal laser microscopy have shown that one of the mBBr-fluorescent proteins is ODF1.

The above findings indicate that proteins labelled by mBBr, including ODF1, are involved in regulation of sperm progressive motility.

Monobromobimane was used both as a chemical agent to interfere with sperm motility and as a marker of sperm thiol status. It exerts a direct, specific effect on thiol groups and exhibits a fast reaction (seconds) with small thiols and a slower reaction with protein thiols (minutes to hours, Kosower and Kosower 1987). In rat spermatozoa, mBBr reduces sperm motility after 10 min of incubation and binds the N-terminal domain of ODF1 (Cabrillana *et al.* 2011). These findings suggest that protein thiols and ODF1 are involved in sperm motility. However, to analyse changes in progressive motility, human spermatozoa are more appropriate than rat spermatozoa.

Binding of DTT thiols to the mBBr reactive site blocks the effect of mBBr on sperm motility. This finding suggests that mBBr binds to protein thiols that are necessary to sperm motility. As we reported previously, the effect of mBBr on sperm motility was abolished by a previous incubation of mBBr

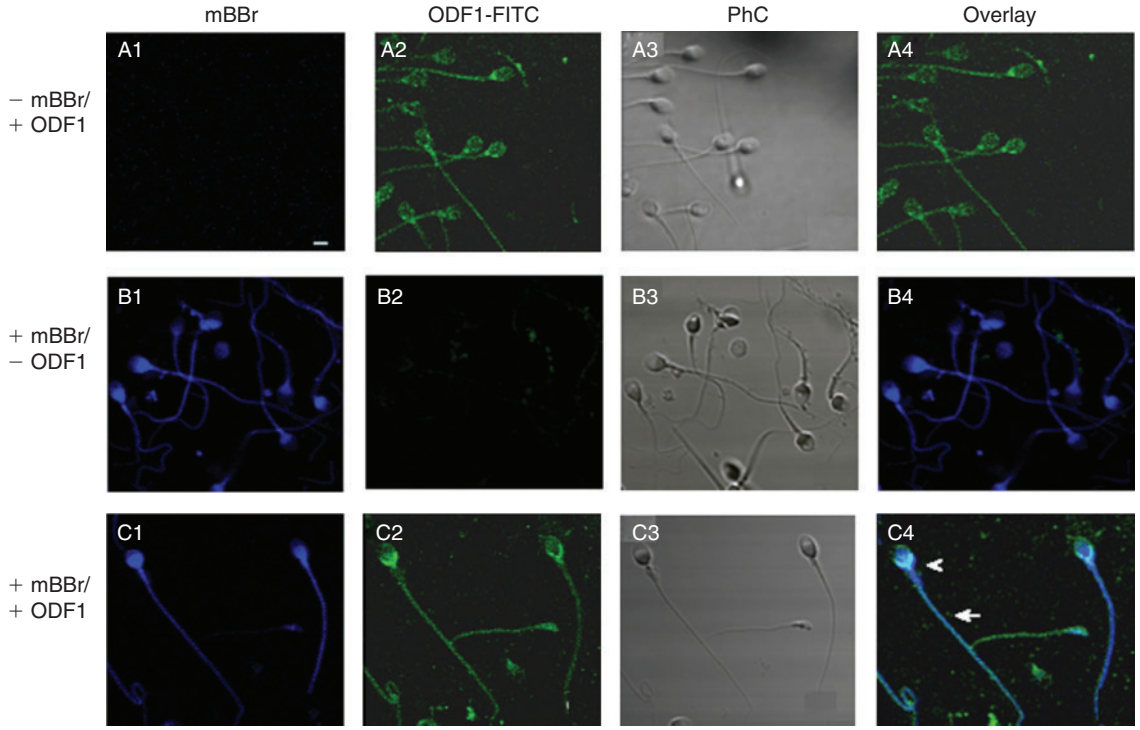

Fig. 3. Colocalisation of mBBr staining with ODF1. Confocal fluorescence micrographs of spermatozoa from normozoospermic men. (*a1*, *b1*, *c1*) Blue fluorescence: location of mBBr affinity proteins. (*a2*, *b2*, *c2*) Green fluorescence: FITC-labelled anti-ODF1. (*a3*, *b3*, *c3*) Phase contrast (PhC) images. (*a4*, *b4*, *c4*) Overlay of blue and green fluorescence signals. (*c4*) Arrow and arrowhead: colocalisation of mBBr and FITC signals. Images shown are representative of three separate experiments.

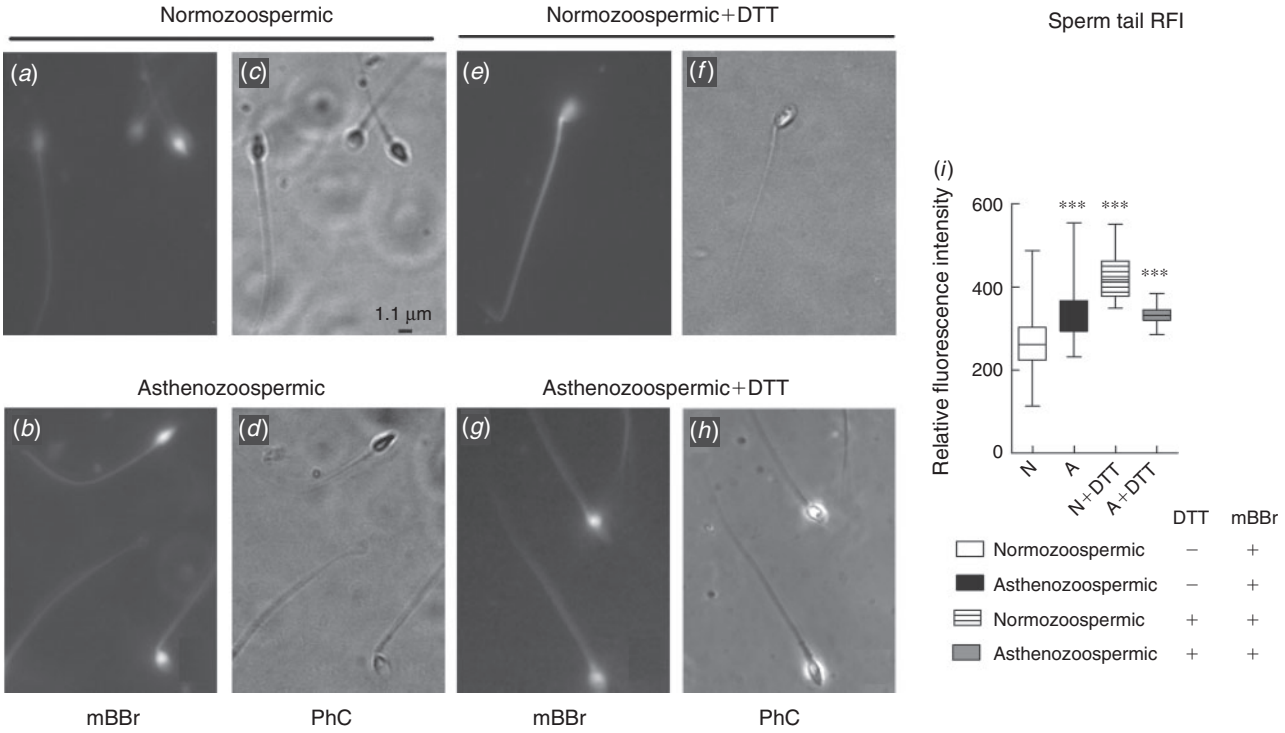

Fig. 4. Monobromobimane fluorescence intensity of sperm tail from normozoospermic and asthenozoospermic men. (*a–h*) Fluorescence microscopy of mBBr-labelled spermatozoa from (*a*, *c*) normozoospermic, (*b*, *d*) asthenozoospermic, (*e*, *f*) normozoospermic+DTT and (*g*, *h*) asthenozoospermic+DTT men; magnification 630× (scale bar = 1.1 µm). (*i*) RFI of mBBr from sperm tail plotted as box (mean) and error bars (minimum to maximum) for the four experimental conditions. RFI values for the other three conditions were compared with normozoospermic RFI. ***$P < 0.001$.

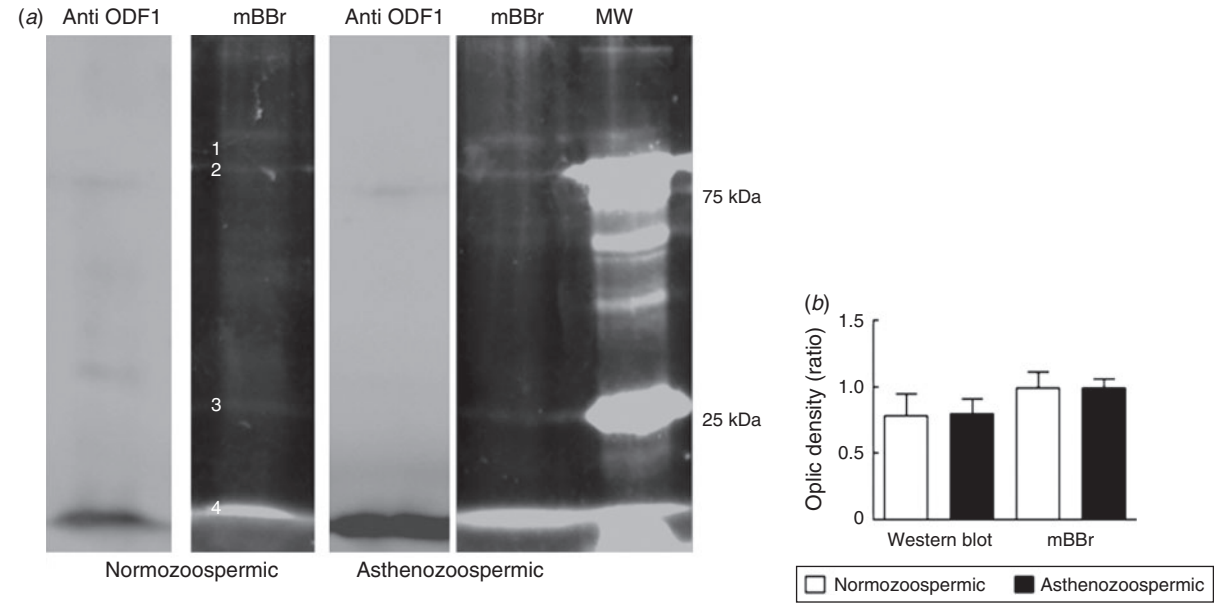

Fig. 5. Identification of mBBr-labelled proteins and western blot analysis for ODF1. (*a*) SDS–PAGE of sperm proteins labelled with mBBr observed under UV light and after blotting with anti-ODF1 antibody, of normozoospermic and asthenozoospermic men. MW, molecular weight. Reference weight markers: 1, 81.28 kDa; 2, 69.18 kDa; 3, 22.9 kDa; 4, 14.79 kDa. (*b*) Ratios of optical density of anti-ODF1 western blot signal and mBBr fluorescence, relative to optical density of Amido Black-stained membrane.

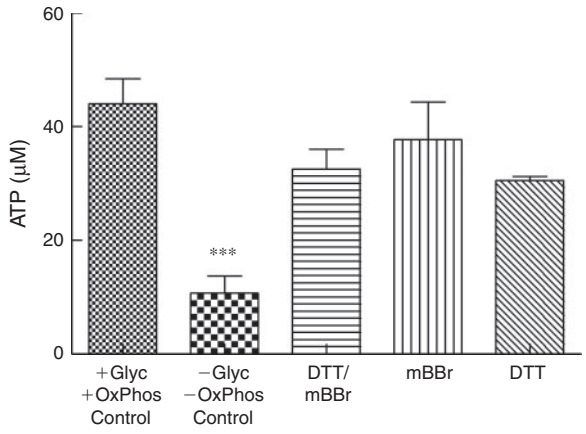

Fig. 6. Effect of mBBr on ATP production. The concentration of ATP was determined in human spermatozoa incubated with: (1) Ham's F10 alone with active glycolysis (Glyc) and oxidative phosphorylation (OxPhos) pathways (+Glyc + OxPhos CONTROL), (2) Ham's F10 without glucose but with 2.52 mM DOG and 40 μM rotenone with inactive Glyc and OxPhos pathways (−Glyc −OxPhos CONTROL), (3) Ham's F10 plus 0.3 mM mBBr co-incubated with 3 mM DTT, (4) Ham's F10 plus 0.3 mM mBBr or (5) Ham's F10 plus 30 mM DTT. Results are expressed as concentration of ATP in mM (mean ± s.e.m.) of three separate experiments. One-way ANOVA was performed to compare data points with control value. ***$P < 0.001$.

with DTT. This methodological tool efficiently blocks the effect of mBBr on sperm motility.

In the present study, VCL and SL were decreased after 10 min of incubation with mBBr, but CP was unaffected; this outcome indicates that the progressive motility was affected by mBBr. Given that mBBr reacts with protein thiols, our attention was focussed on determining which proteins could be involved in this process.

Changes in sperm tail angulation have also been observed (data not shown), suggesting that protein thiols are involved in forward movement of spermatozoa. Changes in tail shape have been correlated with thiol stabilisation and sperm maturation (Cornwall *et al.* 1988; Yeung *et al.* 1999) and the protein adenylate cyclase 10 (ADCY10) has been shown to be involved in flagellar sperm angulations in mice (Ijiri *et al.* 2014).

Several groups have reported relationships between ODF1 and sperm motility. Yang *et al.* (2012) observed that sperm motility was reduced in heterozygous mice with a defect in the N-terminal fragment of ODF1. However, the mechanism whereby ODF1 affects sperm motility is unknown.

In spermatozoa stained with mBBr, both the head and tail become fluorescent. Rat, human and other species have demonstrated involvement of protamines in nuclear mBBr fluorescence (Shalgi *et al.* 1989; Seligman *et al.* 1994; Aleem *et al.* 2008; Ramos *et al.* 2008; de Mateo *et al.* 2011). We showed previously that ODF1 is related to mBBr signals in rat sperm tail (Cabrillana *et al.* 2011). Several authors have described ODF1 in the sperm tail (Oko 1988; Oko and Clermont 1989; Shao and van der Hoorn 1996; Schalles *et al.* 1998; Petersen *et al.* 1999; Shao *et al.* 2001) and Kiersenbaun (2002) detected ODF1 during spermatid differentiation, specifically in the area where the future head was forming. In the present study, FITC-labelled anti-ODF1 and mBBr staining were colocalised in human sperm neck and tail and in the perinuclear zone (Fig. 3*b3*).

Shalgi *et al.* (1989) demonstrated differences in mBBr fluorescence intensity between mature and immature rat

spermatozoa, by direct fluorescence observation and flow cytometry. Our previous study revealed differences in both sperm whole-cell fluorescence and tail signals (Cabrillana *et al.* 2011).

In flow cytometry the entire cell fluorescence is measured; however, this method was applied to quantify the thiol status of only the sperm nuclei because a low percentage of the fluorescence signal comes from the sperm tail (Seligman *et al.* 1991; Ramos *et al.* 2008). Based on these previous findings we measured RFI only in sperm tail from our samples. Microscopic observations permitted a detailed analysis of mBBr fluorescence changes at the sperm tail.

In another study Seligman *et al.* (1994) used mBBr labelling to evaluate thiol status by flow cytometric analysis to measure SH levels and observed increased protamine oxidation in samples of asthenozoospermic men. However, flagellar fluorescence was not analysed.

In another flow cytometric study, de Mateo *et al.* (2011) characterised mBBr-labelled spermatozoa from subfertile patients with oligoasthenoteratozoospermia and demonstrated biphasic distributions reflecting both overoxidation and incomplete thiol oxidation. However, little information is available on sperm tail proteins.

In the present study, the tail fluorescence intensity of mBBr-treated spermatozoa of asthenozoospermic men was higher than that of mBBr-treated normozoospermic men. When spermatozoa of normozoospermic men were preincubated with DTT, high mBBr incorporation in sperm tail proteins was observed under fluorescent microscopy. This finding presumably reflects a high level of thiols in comparison with non-DTT-treated samples. Reduction of spermatozoa of asthenozoospermic men with DTT before mBBr incubation resulted in RFI values similar to those of non-DTT-treated spermatozoa. These results suggest that the spermatozoa from these asthenozoospermic men have incomplete thiol oxidation during epididymal transit.

The molecular weight of ODF1 has been reported as 27 kDa by several groups (Shao and van der Hoorn 1996; Schalles *et al.* 1998; Petersen *et al.* 1999; Shao *et al.* 2001). On the other hand, antibodies against the synthetic peptide DSVRRDIKKV-DRELRQLRCI, derived from the N-terminal region of the putative protein (raised in rats) bound to a 32-kDa protein band (Gastmann *et al.* 1993). We used a goat polyclonal anti-ODF1 antibody (G-17, sc-27907) raised against a peptide mapped near the N-terminus of human ODF1. This antibody recognised a 14.8-kDa protein band that was mBBr positive. More aggressive treatment is required to obtain cytoskeletal proteins of ejaculated spermatozoa for comparison with those of epididymal spermatozoa. This cleavage of proteins could explain the differences in observed running profiles.

A characterisation of mBBr-labelled proteins by MALDI TOF/TOF has shown that proteins smaller than 33 kDa undergo thiol oxidation during the sperm maturation process in the epididymis and these proteins are associated with the cytoskeleton, energetic metabolism and antioxidant defence (Dias *et al.* 2014). This author also suggested that the stabilisation of ODF and fibrous sheath (FS) is related to the organisation of the energy provider apparatus.

Even though proteins involved in the generation of ATP, like glyceraldehyde-3-phosphate dehydrogenase (GAPDH), are labelled by mBBr, such interaction does not affect the production of ATP. In this sense, Costanstini *et al.* (1995) reported that mBBr does not inhibit ATP production in liver cells. These outcomes reinforce the importance of the thiol status of flagellar proteins in sperm motility.

Taken together, our findings indicate that an appropriate thiol status of flagellar proteins, such as ODF1, is necessary to maintain progressive motility of human spermatozoa. Good motility depends, among other mechanisms, on sperm maturation during epididymal transit. This process involves an equilibrated thiol oxidation in sperm flagellar proteins and when mBBr binds to the remnant thiols sperm progressive motility is inhibited.

More studies are in progress to elucidate the mechanism underlying this effect, including the activity of GAPDH, the roles of flagellar proteins other than ODFs, the outer mitochondrial membrane and fibrous sheath and how the epididymal environment promotes an adequate protein thiol status and good sperm motility.

The difference in the thiol status between normozoospermic and asthenozoospermic men could allow us to develop a test to evaluate possible maturation defects in these patients.

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