

Decreased sperm function of patients with myotonic muscular dystrophy

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Myotonic muscular dystrophy (MMD) is a genetic disease caused by a defective enzyme, myotoninkinase. Male patients with MMD are reported to have reduced fertility. The purpose of this work was to study sperm capacitation and acrosome reaction in the ejaculates of sterile males with MMD and of healthy males (control group). The expression of the specific D-mannose receptors was explored by microscopic examination and by flow cytometry analysis. In addition, the binding patterns of *Pisum sativum* (PSA) lectin to acrosome content and outer acrosomal membrane in the spermatazoa of each group were analysed. Both the capacitation and the acrosome reaction in the spermatazoa of the MMD group were deficient and these findings strongly suggest that these anomalies may account for the sterility of these patients.

Key words: acrosome reaction/dystrophy/D-mannose/spermatazoa

Introduction

Myotonic muscular dystrophy (MMD) is defined as a hereditary, autosomal dominant disease characterized by the development of structural and functional abnormalities of the muscle membrane protein myotoninkinase involved in protein phosphorylation (Roses and Apple, 1973, 1974). The mutation that causes myotonic dystrophy is the expansion of the CGT triplets at the 3' end of the gene, that, in turn, is located on chromosome 19 (19q12) (Buxton *et al.*, 1992; Harley *et al.*, 1992).

The mutated gene expresses an abnormal muscle membrane protein kinase and consequently MMD patients present symptoms and signs of muscular, central nervous system and endocrine disorders. Respectively these may include hypotonia of striated and smooth muscle and cardiac muscle conduction defects, ophthalmological pathologies such as cataracts and mental retardation due to endocrine deficiencies. The wide range of the qualitative and quantitative variations of the clinical findings of MMD patients depends on the extent of gene mutation in the different patients (grades I, II, III, IV).

In addition, patients with any of the clinical forms of MMD, but especially males, are reported to have reduced fertility (Harper, 1989). Testicular tubular atrophy in patients with severe forms of this disease is described (Harper, 1989) but according to the World Health Organization (WHO) criteria, some patients with a history of sterility have normal seminal parameters. However, in our research of the literature we found no report in which sperm function in MMD patients had been explored.

This work was designed to study firstly, the capacitation of spermatazoa and, secondly, the acrosome reaction in ejaculates of sterile males with myotonic dystrophy to see if defects in these processes can help explain the reduced fertility of MMD patients.

Materials and methods

Study groups

The patient group consisted of five males with MMD with histories of sterility diagnosed clinically and genetically. All the patients were informed of the purpose of the study and gave their consent. The control group consisted of 10 males with proven fertility who had attended the Reproduction Unit to undergo vasectomy; all had at least two children, one of whom was <1 year old.

Selection of patients

The neurological studies carried out by the Neurology Service were based on patients' clinical features, history and profiles of muscular symptoms. Additional tests were made to determine the degree and extent of muscular weakness, weight loss, myotonia, osteotendon reflexes and both cardiac and circulatory system profiles. We also carried out ophthalmological tests, skeletal and endocrine tests. Several patients, at the discretion of the examiner, received any other tests considered necessary. The results of these tests were used to allocate the patients to their appropriate clinical group: I (symptomatic) and II (slight symptoms like baldness, early cataract or slight myotonia, or both). Patients with more severe conditions were excluded from the study because they had testicular atrophy and this condition would have distorted the results. At the time of the study all patients had a current history of sterility.

The molecular genetic study used DNA obtained from 10–20 ml of peripheral blood. DNA was isolated by the classical method of enzyme digestion with K proteinase and subsequent purification with phenol. Subsequently, the DNA expansion zone was examined by the Southern blot test, which used the restriction enzymes Sac I, Pst I, Eco RI, and Bam HI. After hybridization with the p5B1.4 probe (kindly provided by Dr K.Johnson, Westminster School of London University, UK) marked with dUTP digoxigenin, the reaction was developed by chemiluminescence (Boehringer Mannheim, Germany).

Tests of sperm function

After the samples were extracted, collected and treated, according to the recommendations (WHO, 1992), an analysis of the ejaculates of

both study groups was carried out. Only donors displaying normal semen analysis were used as controls in the present study [volume >2 ml; sperm count >20×10⁶ cells/ml; motile sperm type Q(+++) (WHO, 1992) >25%].

In the study group, specimens in which sperm concentrations were too low were excluded from the study. The seminal parameters (mean ± SD) of the five patients selected were within normal ranges: volume: 3.2 ± 1.3 ml; concentration: 78 ± 25×10⁶ cells/ml; motility type Q(+++): 27 ± 10%; vitality: 88 ± 21%. The 'swim-up' method was used to select spermatozoa with movement Q(+++) in Ménézo B2 medium (INRA Ménézo B2, BioMerieux SA, Marcy-l'Etoile, France).

Capacitation

After 'swim-up', an aliquot of the supernatant with a concentration of 1×10⁶ cells/ml was exposed to capacitating conditions and incubated for a minimum of 3 h at 37°C in Ménézo B2, aerated with CO₂ 5%. Sperm capacitation was measured by detecting and measuring the lectin-like specific binding sites of D-mannose by the sperm membrane in the pre- and post-capacitation phases (Benoff *et al.*, 1993b,c).

Sperm samples from both study groups were incubated for 30 min at 37°C in an atmosphere of 5% CO₂ (Tesarik *et al.*, 1991) with fluorescein-labelled mannose albumin (man-BSA-FITC, Sigma A-7790; Sigma Chemical Co., St Louis, MO, USA) in a final concentration of 100 µg/ml for epifluorescence microscopy study of binding patterns (Olympus IM, Olympus, Tokyo, Japan), or 20 µg/ml for flow cytometry analysis (Ortho Citoron Absolute, ORTHO Diagnostics System, Johnson & Johnson Co., Madrid, Spain). After washing the sperm samples with phosphate-buffered solution (PBS; Gibco, Paisley, UK) and centrifuging for 10 min at 300 g the spermatozoa were spread on microscope slides and left to dry in darkness at room temperature. For the flow cytometry analyses, the washed gametes were resuspended in PBS and their concentrations were adjusted to 1×10⁶ cells/ml.

Control samples were incubated at the same time with BSA-FITC (Sigma) to adjust the background fluorescence and D-mannose oligosaccharide (M-4625, Sigma) was used as the competitive inhibitor of the D-mannose binding sites to eliminate the possibility of non-specific linkages.

The binding patterns in both pre-capacitation (fresh samples) and post-capacitation (capacitated) samples were studied by fluorescence microscopy. Following previously described methods (Benoff *et al.*, 1993b,c), two types of distribution patterns were examined: pattern I: fluorescence of the sperm neck; pattern II: fluorescence of the sperm membrane of the acrosome and equatorial region or neck, or of both. These two patterns were mutually exclusive, i.e. they never co-existed in the same preparation.

The parameters considered after flow cytometry analyses of the suspensions of spermatozoa were: percentage of fluorescent cells in each sample and the mean fluorescence channel (MFC) displayed by the fluorescent cell populations.

Acrosome reaction

Study of the acrosome reaction followed a previously described method (Ramirez *et al.*, 1994). To samples of spermatozoa incubated under capacitating conditions were added either progesterone (Sigma) to give a final concentration of 1 µl/ml, or calcium ionophore to give a final concentration of 20 µl/ml (Ca²⁺ ionophore A23187, C-7522, Sigma). After washing three times with PBS, a drop of each pellet was spread on a microscope slide and allowed to dry before being fixed in methanol for 30 s and subsequently incubated with the *Pisum sativum* lectin (PSA-FITC; Kem en Tec, Menarini, Spain).

The PSA lectin links the D-mannose oligosaccharide moieties of

Table I. Lectin-like D-mannose receptors expression in non-capacitated and capacitated spermatozoa from patients with myotonic muscular dystrophy (MMD) and subjects in the control group

	Non-capacitated		Capacitated	
	% (+) ^a	CFM ^b	% (+) ^a	CFM ^b
Control (n = 10)	4.2 ± 1.7	116.9 ± 7.3	30.7 ± 5.4	114.3 ± 12.6
MMD (n = 5)	3.9 ± 2.3	113.7 ± 6.8	6.4 ± 1.2 ^c	110.1 ± 16.3

^aPercentage of spermatozoa showing fluorescence (mean ± SD).

^bMean fluorescence channel.

^cP < 0.01.

the acrosome external membrane and the contents of the acrosomal matrix. Those spermatozoa that displayed the acrosome reaction were seen to have lost their matrix and their acrosome external membrane and their equatorial band was either stained or not stained. Consequently, reacted spermatozoa were easily differentiated and counted to give the percentages of reacted and stained spermatozoa before and after treatment.

The percentage of spermatozoa that displayed spontaneous acrosome reaction was found by analysis of an aliquot of spermatozoa incubated under the same conditions, but without ionophore or progesterone.

Statistical methods

The data shown are means (±SD). The Mann-Whitney non-parametric test was used to analyse the data.

Results

After fluorescence microscopy of the non-capacitated (pre-capacitation) sperm smears, no differences were found between the linking patterns of the two study groups. Non-capacitated spermatozoa showed pattern I, i.e. representing a weak expression of the membrane of the neck. However, after the incubation under capacitation conditions (post-capacitation spermatozoa), the linking patterns observed were different between the two groups. While the capacitated spermatozoa of the control group displayed increased fluorescence and pattern II, i.e. representing the D-mannose binding sites that progressively migrated to the membrane surface of the acrosomal and equatorial regions, spermatozoa of the MMD group did not show pattern II.

Table I shows the results of the flow cytometry analyses. The expression of the D-mannose binding sites in non-capacitated spermatozoa was similar in both study groups; no significant differences were found between the percentages of spermatozoa showing fluorescence (4.2 versus 3.9%, not significant). The mean fluorescence channels were also similar in both cases.

However, the percentages of fluorescent cells obtained after flow cytometry analyses following incubation of the gametes under capacitating conditions showed statistically significant differences between those of healthy control subjects and MMD patients (30.7 versus 6.4%; P < 0.01) (Table I). Only a small percentage of the spermatozoa of the MMD group showed fluorescence after incubating under capacitation condi-

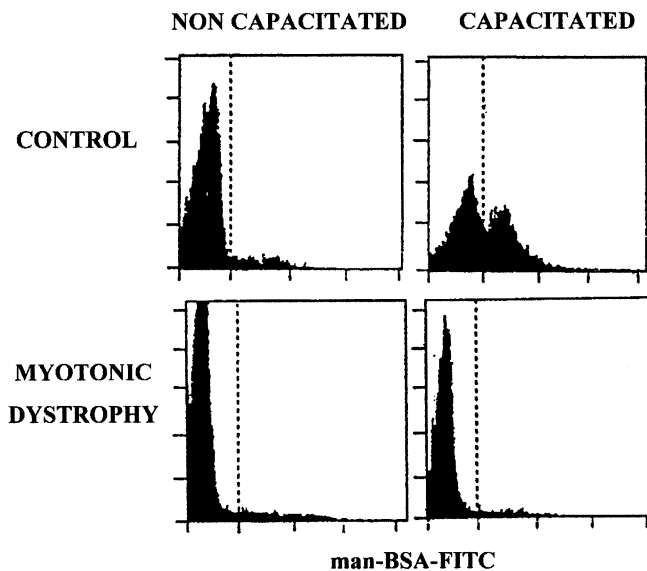


Figure 1. Fluorescence histogram of specific D-mannose binding in the control and myotonic dystrophy groups before and after capacitation.

Table II. Evaluation of spontaneous and induced acrosome reaction (AR) in myotonic muscular dystrophy (MMD) by the *Pisum sativum* method

	Spontaneous AR ^a	Induced AR	
		IC A23817 ^b	Progesterone ^b
Control group (n = 10)	6.1 ± 3.7	29.4 ± 10.3	27.1 ± 7.3
MMD (n = 5)	4.6 ± 2.4	10.1 ± 3.3 ^c	13.6 ± 6.7 ^c

^aPercentage of spermatozoa that showed spontaneous acrosome reaction (mean ± SD).

^bPercentage of spermatozoa that showed induced acrosome reaction minus % of spermatozoa that showed spontaneous acrosome reaction (mean ± SD).

^cP < 0.01.

tions. Figure 1 shows the fluorescence histograms of the specific D-mannose binding in the two groups before and after capacitation.

Table II shows the results of the acrosome reaction study. The spontaneous acrosome reactions of the two groups were similar. Nevertheless, after exposure to agents that induce the acrosome reaction, either after treatment with progesterone or calcium ionophore, significant differences were found between the proportions of reacted spermatozoa of the two groups. The linking patterns of PSA given by fluorescence microscopy represented the percentage of spermatozoa that showed the acrosome reaction.

Discussion

It has been proposed (Benoff *et al.*, 1993c) that the expression of specific binding sites for D-mannose could be used as a specific laboratory method to measure the capacitation of a sperm population instead of the less specific classical methods. Thus, the decreased expression of the D-mannose binding sites

found by microscopic examination and flow cytometric analysis of spermatozoa from the MMD patients studied in the current report revealed that defective capacitation was characteristically associated with MMD.

The expression of specific binding sites for D-mannose of the male gamete is positively correlated with the fertilizing potential of an ejaculate (Tesarik *et al.*, 1991; Benoff *et al.*, 1993b,c; Gabriele *et al.*, 1998) and treatment with D-mannose completely inhibits penetration through the zona pelucida (penetration test) (Mori *et al.*, 1989; Gabriele *et al.*, 1998). It is accepted that the lectin-like binding sites on the membrane surfaces are like a linkage and recognition mechanism for D-mannose residues in the oocyte zona pellucida (Mori *et al.*, 1993).

Similarly, we detected significant decreases in the intensity of the induced acrosome reaction that might also help to explain the decreased sperm fertilizing potential in MMD patients. The fact that the measured responses to induction of the acrosome reaction *in vitro* are correlated with the fertilizing potential of sperm populations (de Jonge, 1994) suggests strongly that the decreased acrosome response which we observed accounts for the sterility of MMD patients.

Further research will be necessary in order to determine the molecular anomalies that explain these alterations. Present knowledge allows us to suggest some probable defects. Sperm cytoplasmic membranes are closely involved in capacitation and acrosome reaction processes. Optimum fluidity of the sperm membrane is essential for the processes of fusion, vesiculation and disappearance of the plasma and acrosomal membranes, which takes place during the acrosome reaction. It has been reported (Benoff *et al.*, 1993a,b,c,d) that the expression of lectin-like specific-binding D-mannose receptors during capacitation is closely correlated with the integrity of the two lipid layers of the cell membrane. Consequently, the location of the receptors between the cytoplasmic and external acrosomal membranes needs optimum fluidity of the lipid layer for these receptors to be expressed correctly. Further, it is well documented that sperm protein phosphorylation increases with capacitation (Visconti and Kopf, 1998) and also forms part of the cascade of processes that leads to acrosome exocytosis (Benoff, 1998). Capacitation depends primarily on phosphorylation of serine and threonine residues (Naz, 1999) while exocytosis also needs tyrosine phosphorylation events.

Our results lead us to suggest that defective sperm capacitation and acrosome loss might be characteristic in sperm specimens of men with MMD. These data provide the first direct evidence for defects in sperm function in these patients.

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