Mitochondria from ejaculated human spermatozoa do not synthesize proteins

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Abstract Sperm motility is dependent on mitochondrial ATP production that relies on the coordinated expression of the mitochondrial and nuclear genomes. It is generally accepted that mammalian ejaculated spermatozoa retain the ability to synthesize mtDNA-encoded proteins but not most of the nuclear ones. This implies an asynchronous regulation of the oxidative phosphorylation-related genes encoded by each genome. Trying to investigate this issue, we unexpectedly found that ejaculated human spermatozoa do not synthesize mtDNA-encoded proteins. Moreover, we estimated that the discrepancy between our observations and those published elsewhere was due to a chloramphenicol-sensitive protein synthesis attributed to mitochondria that instead corresponds to contaminating bacteria.

Key words: Mitochondrial DNA; Spermatozoon; Spermatogenesis; Protein synthesis; Mitochondrion

1. Introduction

Mitochondria are arranged around the axial filament in the middle piece of spermatozoa and one of their main roles is the supply of energy for the flagellar activity by oxidative phosphorylation (OXPHOS). Therefore, since a small but fundamental set of proteins required to generate a fully functional OXPHOS system is encoded by mtDNA, its expression is needed for the correct development and function of spermatozoa. In this sense, it is known that mtDNA alterations can promote a decrease in sperm motility [1–8]. Moreover, mtDNA common variants (haplogroups), thought to be non-pathological, can determine the motility performance of the spermatozoa [9].

OXPHOS system biogenesis requires the concerted expression of two genomes, nuclear and mitochondrial. In addition, a fully differentiated spermatozoon has shut down most of its biosynthetic capacity [10] and therefore it is of interest to determine how mtDNA expression is adapted to this situation and, in particular, if mitochondria maintain their synthetic protein activity after ejaculation. An early report proposed an active mitochondrial protein synthesis activity in ejaculated bull sperm [11,12]; additional evidence supported this observation in a variety of mammalian species: human [13], ram [14,15] and mouse [16]. Therefore, an asynchrony between the expression of nuclear and mtDNA OXPHOS-encoded genes could potentially occur in spermatozoa.

During our investigation aimed to understand the biogenesis of the OXPHOS system in spermatozoa, we have reevaluated the protein synthesis activity of mitochondria in ejaculated human sperm. In contradiction with previous results, we found no significant protein synthesis activity by spermatozoan mitochondria. Moreover, by a series of experiments monitoring the incorporation of radiolabeled methionine in the presence of different protein synthesis inhibitors, and a variety of antibacterial agents, we concluded that the discrepancy between previous results and those presented here is probably due to the wrong attribution to mitochondria of a chloramphenicol (CAP)-sensitive protein synthesis activity, which very likely corresponds to bacteria contaminating the sperm preparations.

2. Materials and methods

2.1. Preparation and characterization of human spermatozoa

Samples from four healthy donors from Zaragoza (Spain) were collected by masturbation under hygienic conditions, after a period of sexual abstinence of 3–5 days. The samples were allowed to liquefy for 30 min at 37°C and analyzed according to World Health Organization recommendations. Within a period of 2 h, the volume of the ejaculate was measured and the number and percentage of motile spermatozoa was evaluated [17]. Spermatozoan morphology was assessed by light microscopy to select donors with normal sperm parameters. All the samples utilized in this study were collected after consent of the donors and under the supervision of the 'Comité de Ética de Investigación Clínica' (Universidad de Zaragoza) with approval number 575.

2.2. Semen fractionation, collection and cell preparation

In order to reach the desirable concentration of round cells/ml or progressive spermatozoa/ml in each final aliquot, we pooled semen samples from four healthy individuals and the pool was fractionated at 1000×*g* for 13 min in a discontinuous Percoll gradient. Then, four fractions corresponding to 90, 47, 25% of Percoll and the fraction over the gradient that we called Rest were collected. The 90 and 47% Percoll phases concentrate highly progressive spermatozoa [18], and were collected, pooled and diluted in 5 ml Dulbecco's modified Eagle's medium (DMEM) without methionine. The 25% Percoll phase and Rest, containing low amounts of non-progressive spermatozoa

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Abbreviations: CAP, chloramphenicol; DMEM, Dulbecco's modified Eagle's medium; EME, emetine; OXPHOS, oxidative phosphorylation; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis

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Table 1
Protein synthesis estimated as TCA-insoluble radioactivity of the spermatozoan preparations under the indicated conditions

Pretreatment	Non-antibiotic-pretreated		Antibiotic-pretreated							
	Penicillin G (100 μg/ml) during protein labeling		Penicillin G (100 μg/ml), 3 h			Streptomycin (100 μg/ml), 1 h			Nalidixic acid (10 μg/ml) 1 h		
Protein synthesis inhibitor	None	EME	CAP	None	EME	CAP	None	EME	CAP	None	EME	CAP
Spermatozoa (cpm/μg protein)	6735 ± 1524	6550 ± 331	1358 ± 45	598 ± 104	723 ± 143	343 ± 131	529 ± 108	355 ± 95	745 ± 155	253	134	173
Round cells (cpm/μg protein)										2536	280	1160

and mainly non-sperm cells (round cells), were collected, pooled and diluted in a similar way. Each preparation of spermatozoa or round cells was pelleted by centrifugation at $1000 \times g$ for 13 min and resuspended to a final concentration of 10×10^6 or 100×10^6, respectively, of round cells/ml or progressive spermatozoa/ml in DMEM without methionine.

2.3. Cell lines

The human transmitochondrial cell line pT3 [19] was grown in DMEM supplemented with 5% fetal bovine serum.

2.4. Measurement of mitochondrial protein synthesis in intact cells

The labeling of mtDNA-encoded proteins in intact cells was performed with [^{35}S]methionine, following the protocol described elsewhere with small modifications [20]. Briefly, mitochondrial protein synthesis was monitored by the incorporation of 100 μCi of Pro-Mix™ [^{35}S]methionine (purchased from Amersham Life Science, Buckinghamshire, UK) in the presence of 40 μg/ml of CAP or 100 μg/ml of emetine (EME, emetine hydrochloride, Sigma, St. Louis, MO, USA), which specifically inhibit mitochondrial or cytoplasmic protein synthesis, respectively. Where indicated, cells were treated before or during the assay with additional antibiotics: 100 μg of penicillin G/ml, 100 μg of streptomycin/ml or 10 μg of nalidixic acid/ml (Sigma).

After incubation, the excess of non-incorporated radioactive amino acid was eliminated by washing the cells twice with 2 ml of phosphate-buffered saline. Finally each cell sample was resuspended in 50 μl of 10 mM Tris–HCl pH 7.4, 0.1% sodium dodecyl sulfate (SDS), 1 mM phenylmethylsulfonyl fluoride. Trichloroacetic acid (TCA) precipitation and SDS–polyacrylamide gel electrophoresis (PAGE) analysis of the labeled products was performed as described elsewhere [20].

3. Results

3.1. [^{35}S]Methionine is incorporated in proteins by human progressive spermatozoa preparations and in the presence of penicillin G

Premkumar and Bhargava [11,12] stated that bull spermatozoan mitochondria were able to incorporate radiolabeled amino acids in proteins (TCA-precipitable products) based on the following arguments: (1) most of the cells in the semen sample were spermatozoa, (2) the incorporation was CAP-sensitive, and (3) the presence of penicillin G during the assay would prevent the incorporation of the radiolabeled amino acid by bacteria.

In addition to spermatozoa, semen contains a number of other cells (spermatozoan precursors, leukocytes, Sertoli cells, squamous epithelial cells and enucleated residual bodies) collectively named round cells [21]. Some of these non-sperm cells are necrosed, but others are still alive and active. To precisely address the potential ability of mature sperm cells to synthesize proteins, it is necessary to separate them from non-sperm cells. Fractionation of semen into a discontinuous Percoll gradient is a convenient technique that allows the separation of the different types of cells found in semen. Most of the progressive spermatozoa sediment preferentially into the 90% Percoll phase; a lower proportion of progressive and most of the non-progressive spermatozoa sediment into the 47% Percoll phase; non-progressive spermatozoa and a proportion of round cells sediment into the 25% Percoll phase and most of the round cells and, especially, residual bodies remain on top of the gradient [18].

Therefore, we repeated the experiment performed by Premkumar and Bhargava [11,12] but eliminating almost completely the non-sperm cells. For that purpose, we assayed protein synthesis of human ejaculated progressive spermato-

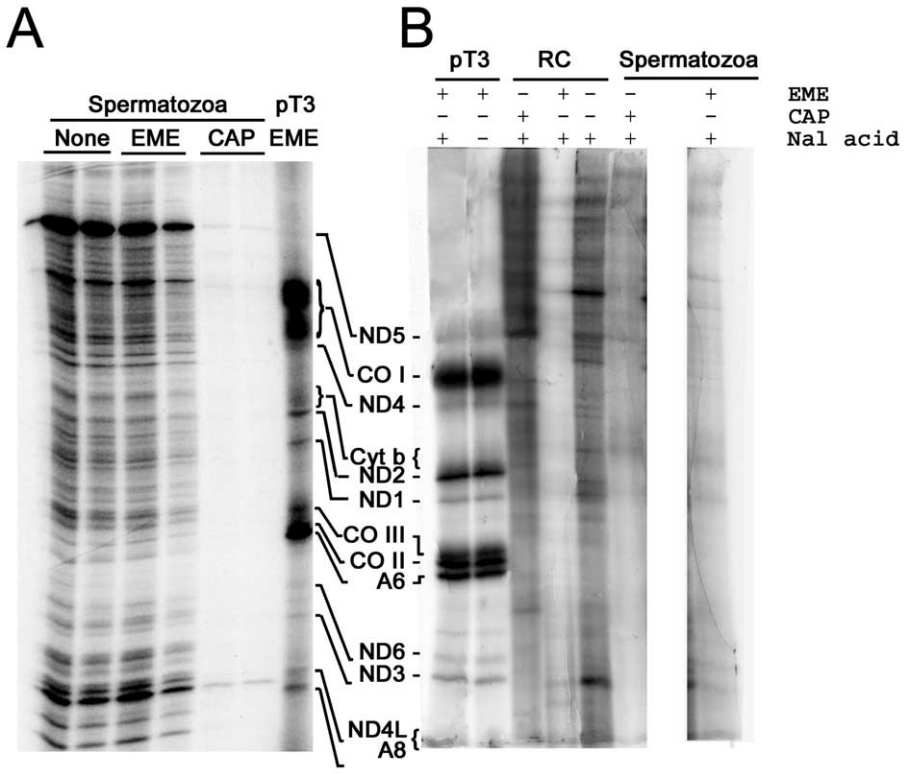

Fig. 1. Qualitative analysis of the proteins synthesized by the sperm cell preparations. A: Autoradiogram showing the pattern of [^{35}S]methionine-labeled proteins by 100×10^6 human progressive spermatozoa/ml in 1 h and in the presence of penicillin G (100 μg/ml) only during the labeling. Line labeled as pT3 corresponds to a similar pattern obtained by a control human cell line labeled with [^{35}S]methionine for 1 h in the presence of EME to allow only the labeling of mtDNA-encoded proteins. B: Autoradiogram showing the pattern of [^{35}S]methionine-labeled proteins during 6 h and by 10×10^6 round cells/ml or 100×10^6 human progressive spermatozoa/ml in the presence of 10 μg/ml nalidixic acid (Nal acid) and with 1 h of incubation prior to labeling. It is also shown that the nalidixic acid does not influence mitochondrial protein synthesis of the control cell line pT3. Symbols are as follows: COI, COII, and COIII, subunits I, II, and III of cytochrome *c* oxidase; ND1, ND2, ND3, ND4, ND4L, ND5, and ND6, subunits 1, 2, 3, 4, 4L, 5, and 6 of the respiratory chain NADH dehydrogenase; A6 and A8, subunits 6 and 8 of H^+-ATPase; Cyt b, apocytochrome *b*.

zoa, collected in the 47 and 90% Percoll pooled fractions, in the presence of either 40 μg/ml of CAP or 100 μg/ml of EME. In addition, 100 μg/ml of penicillin G was added during the labeling. In that way, we reproduced the conditions described originally [11,12]. When protein synthesis was then monitored by the incorporation of [^{35}S]methionine into TCA-insoluble proteins (Table 1), we obtained a result that was coincident with that published in the early 1970s [11,12]. Thus, spermatozoa preparations were able to incorporate the labeled amino acid in TCA-insoluble proteins. In addition, this incorporation showed a very different sensitivity to cytoplasmic- (EME) or mitochondrial-specific (CAP) protein synthesis inhibitors. As described by Premkumar and co-workers, most of the incorporation was CAP-sensitive (83%).

Mitochondrial DNA encodes only 13 polypeptides that can be resolved in a SDS–PAGE gradient gel [20]. To evaluate qualitatively the proteins synthesized by the spermatozoa preparation in the presence of EME, we analyzed the labeled products by SDS–polyacrylamide gradient electrophoresis. To our surprise, the electrophoretic protein patterns of incorporated [^{35}S]methionine were completely different between the human mitochondrial reference pattern (pT3 cells) and that from proteins labeled by the spermatozoa preparations. Moreover, it was impossible to identify a single mtDNA-encoded protein (Fig. 1A). The most likely explanation for this unexpected observation was that, since their synthesis was CAP-sensitive, the proteins labeled by the spermatozoa preparation probably derive from contaminating bacteria.

3.2. *[^{35}S]Methionine incorporation by human progressive spermatozoa preparations is mainly due to contaminating bacteria*

If the above interpretation is correct it would imply that the presence of penicillin G at 100 μg/ml during the labeling period is insufficient to prevent bacterial protein synthesis. To test this possibility, we performed experiments treating the spermatozoa samples with different antibiotics: (a) 3 h pretreatment with 100 μg/ml penicillin G/ml at 37°C; (b) 1 h pretreatment in the presence of 100 μg/ml of streptomycin; (c) 1 h of pretreatment in the presence of 10 μg/ml of nalidixic acid.

All these pretreatments were intended to kill and eliminate the bacteria suspected to be present in the spermatozoa preparation while keeping the potential biogenetic activity of the spermatozoa. As summarized in Table 1, incorporation of [^{35}S]methionine dramatically drops to negligible values with any of the antibiotic pretreatments. Moreover, SDS–PAGE also failed to detect true mitochondrial proteins in the residual labeling (Fig. 1 and results not shown). In conclusion, systematic elimination of bacteria using a variety of antibiotics that target different biogenetic pathways (wall synthesis by penicillin G, protein synthesis by streptomycin and DNA metabolism by nalidixic acid) almost completely eliminates the CAP-

sensitive protein synthesis activity of human ejaculated spermatozoa. Although we believe the CAP-sensitive protein synthesis activity is entirely due to bacteria it remains possible that true mitochondrial protein synthesis occurs in the non-sperm cells present in semen.

We investigated this possibility by performing protein synthesis assays with the round cell-enriched preparation obtained from the Percoll gradient described above (25% Percoll phase and seminal plasma) to eliminate progressive spermatozoa, and pretreated for 1 h with nalidixic acid to kill bacteria. It should be remembered that the round cell fraction contains mainly cell debris. As shown in Table 1 protein synthesis by round cell-enriched preparations showed high EME sensitivity, indicating that it is mainly of cytoplasmic origin. Moreover, when analyzed by SDS–PAGE no mtDNA-encoded proteins could be identified (Fig. 1B).

4. Discussion

Previous studies reported RNA and/or protein synthesis in spermatozoa of several mammalian species: bull [11,12], human [13], mouse [16] and ram [14,15]. In spite of the precautions taken by the investigators to prevent bacterial contamination, we have shown that they are insufficient to avoid bacterial protein synthesis. In the case of the earliest report on bull spermatozoa, the presence of biogenetically active bacteria is also revealed by the analysis of the nature of the RNA synthesized by the sperm preparations [12]. Thus by centrifugation, de novo synthesis was estimated of 23S and 16S rRNAs, wrongly interpreted as mtDNA-encoded rRNAs, since we know today that the sedimentation coefficients of mammalian mitochondrial rRNAs are 16S and 12S and those of the bacterial rRNAs are 23S and 16S. This observation reinforces our interpretation suggesting the presence of biogenetically active bacteria as responsible for the CAP-sensitive protein synthesis activity. Our findings demonstrate that human ejaculated spermatozoa are not capable of synthesizing proteins, being biogenetically inactive and unable to modify their OXPHOS capacity by de novo synthesis of respiratory complexes once differentiated. Our results are in good agreement with the proposed shut-down of mtDNA transcription during spermatogenesis [22,23].

The observation reported here has two main implications. First, the postulated asynchrony in the regulation of mtDNA- and nuclear-encoded OXPHOS genes can be discarded. And second, the biogenesis of the OXPHOS system along the maturation of the spermatozoa takes place and becomes fixed well before its function could be evaluated. As a consequence of this, when motility is activated after maturation, increasing the need for a high energy supply, a compensatory biogenetic response is not allowed. Therefore, an unknown quality control mechanism may operate, specific for the spermatozoan OXPHOS system, and different from what we know for other tissues, in particular muscle. In conclusion, the OXPHOS capacity of spermatozoa is achieved along the spermatogenesis before they become fully motile.

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