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Isolation and Characterization of Mitochondria from Turkey Spermatozoa

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ABSTRACT: A procedure was developed to rapidly isolate functional, intact mitochondria from turkey spermatozoa. Semen was collected from turkeys, pooled, and centrifuged to remove spermiophages and other cells. The sperm cells were then mechanically disrupted with a Dounce homogenizer, sonicated, and centrifuged using a discontinuous Percoll gradient. Electron microscopy revealed morphologically intact mitochondria. The isolated mitochondria exhibited cytochrome oxidase activity, oxygen consumption, and were stained by rhodamine 123, a fluorescent stain specific for functional mitochondria in eukaryotic cells. Mitochondrial DNA (mtDNA) was isolated and purified, and the genome was determined to be 16.457 ± 0.07 kbp. Restriction fragment patterns were identified using the endonucleases EcoR1, HindIII, and BamH1. Mitochondrial DNA was also purified from turkey liver and testis, and no differences in the restriction enzyme patterns were found between somatic and germ cell mtDNA. It is concluded that mitochondria can be isolated from spermatozoa for metabolic or genetic study.

Key words: Turkey, spermatozoa, mitochondria, mitochondrial DNA.

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Mitochondria provide the cell with the majority of ATP through the aerobic respiration of oxidative phosphorylation. For this reason, procedures to isolate mitochondria from organs such as the liver (Schnaitman and Greenawalt, 1968; Bustamante et al, 1977) for metabolic research have been developed. These procedures require the animal to be sacrificed to obtain tissue for isolation of mitochondria. Spermatozoa represent ideal cells to isolate mitochondria because they have scant cytoplasm and condensed nuclear DNA, which easily separates from mitochondria on the midpiece. Furthermore, many semen samples can be collected without having to harm or sacrifice the animal.

In mammals, morphological and structural changes occur in mitochondria during spermatogenesis (Fawcett, 1970; De Martino et al, 1979; Hecht and Bradley, 1981). The mature spermatozoal mitochondria have different polypeptides and density than somatic cell mitochondria (Pallini, 1979; Hecht and Bradley, 1981). It has been shown that spermatozoal mitochondria have specific isozymes such as lactate dehydrogenase and cytochrome c (De Domenech et al, 1972; Goldberg et al, 1977). The mitochondrial DNA (mtDNA) of spermatozoa may also differ from somatic cell mtDNA as it has been shown that ram spermatozoal mtDNA have a different buoyant density, base composition, and contour length than somatic mtDNA (Fisher et al, 1977; Bartoov and Fisher, 1980). A procedure to isolate spermatozoal mitochondria would provide for the study of the unique aspects of mitochondrial function in spermatozoa and could facilitate the study of differences between germ and somatic cell mitochondria.

The goals of the present study were to isolate turkey sperm mitochondria for the purpose of studying their viability and metabolism. In addition, mtDNA was isolated and restriction enzyme patterns were identified using the endonucleases EcoR1, HindIII, and BamH1. The restriction enzyme pattern of sperm mtDNA was also compared with that of liver and testis cells.

Materials and Methods

Isolation of Mitochondria

Semen was collected from turkeys (*Meleagris gallopavo*) by abdominal massage (Burrows and Quinn, 1937), pooled, and diluted 1:1 with 1.07 g/ml Percoll (Sigma Chemical Co, St. Louis, Missouri). The samples were then centrifuged in a microcentrifuge (model 235A, Fisher Scientific Co, Pittsburgh, Pennsylvania) at 12,000 × *g* at room temperature for 2 minutes to remove spermiophages that remained in the supernatant. The pelleted

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sperm were resuspended in phosphate-buffered saline (PBS, Gibco BRL, Gaithersburg, Maryland), then overlaid on 1.045 g/ml Percoll and centrifuged at 2,500 × *g* for 30 minutes at 10°C in a Sigma 3K12 centrifuge with a swinging bucket rotor (B. Braun Biotech, Inc, Allentown, Pennsylvania). The supernatant, which contained remaining spermiophages and other mononuclear cells, was removed, and the pelleted sperm were again resuspended in PBS. They were placed in a Dounce homogenizer on ice and mechanically disrupted using eight strokes with the large clearance pestle (0.089 mm, 0.165 mm) followed by eight strokes with the small clearance pestle (0.0254 mm, 0.0762 mm). The disrupted sperm were then placed in a centrifuge tube on ice and sonicated four times at 50% setting, 15 seconds each with a Vibra Cell Sonicator (Sonics & Materials, Inc, Danbury, Connecticut), with a 15-second rest between sonications. The resulting suspension was overlaid on 1.055 g/ml Percoll and centrifuged at 3,000 × *g* for 45 minutes at 10°C in the Sigma 3K12 centrifuge. The mitochondria formed a band at the PBS/Percoll interface, while other sperm components pelleted at the bottom of the tube. The mitochondria were removed with a pipet and pelleted in microfuge tubes by centrifuging at 12,000 × *g* for 2 minutes at room temperature. The mitochondria were then resuspended with PBS and recentrifuged to remove any remaining Percoll and resuspended in the appropriate solution for further study.

The procedure to isolate mitochondria from liver and testis was based on that of Cantatore et al (1988). Turkeys were sacrificed by sodium pentobarbitol overdose, and the liver and testis were removed and transported under sterile conditions. The tissue was minced in 0.25 M sucrose and homogenized as described above. After mixing, the suspended cells were passed through a sterile 150-mesh screen, then overlaid on 0.44 M sucrose and centrifuged at 500 × *g* for 10 minutes at 10°C. The supernatant was removed and centrifuged at 9,000 × *g* for 30 minutes at 10°C. The resulting pellet was resuspended in PBS, overlaid on 1.055 g/ml Percoll, and centrifuged at 3,000 × *g* for 45 minutes at 10°C. A band of mitochondria formed at the PBS/Percoll interface. The mitochondria were removed with a pipet and pelleted in microfuge tubes by centrifuging at 12,000 × *g* for 2 minutes at room temperature. They were then resuspended with PBS and recentrifuged to remove any remaining Percoll and resuspended in the appropriate solution for further study.

Transmission Electron Microscopy

Mitochondria were washed with Millonig's phosphate buffer (Millonig, 1962) and centrifuged to pellet the sample. They were fixed 3 hours with 2% glutaraldehyde then 2 hours with 2% osmium tetroxide (phosphate buffered). Dehydration with stepwise ethanol concentrations ending with 100% ethanol followed the fixations. The mitochondria were embedded in Poly/Bed 812-Araldite. Selected areas were ultrathin sectioned (90–99 nm) with a diamond knife, placed on copper grids, and stained with 3% uranyl acetate for 15 minutes and 0.3% lead citrate for 30 seconds. These sections were examined with a JEOL 100-C electron microscope using an acceleration voltage of 60 kV.

Fluorescence Microscopy

Use of the dye rhodamine 123 as a measure of mitochondrial function is based on Johnson et al (1980, 1981). Purified rhodamine 123 (Sigma Chemical Co) was dissolved in RPMI medium 1640 (Gibco Laboratories, Grand Island, New York) at a concentration of 20 μg/ml. Washed mitochondria were resuspended in the RPMI containing the rhodamine 123 and incubated for 3 hours at 41°C. The mitochondria were pelleted at 12,000 × *g* for 2 minutes, washed, and resuspended in PBS. Stained mitochondria were examined and photographed under epifluorescent illumination at an excitation wavelength of 400–440 nm on a Nikon Optiphot microscope (Nikon Inc, Instrument Group, Garden City, New York) equipped with a CFN Plan Objective (× 100 oil) and UFX-IIA exposure system. Sperm cells were stained with rhodamine 123 (20 μg/ml) for 3 hours at room temperature. Then, 50 μg/ml of acridine orange was added, and the sample was incubated for an additional 5 minutes. The sperm were centrifuged, washed with PBS, and placed on a slide for observation.

Cytochrome Oxidase

Validity and basic concepts of the cytochrome oxidase assay were obtained from Wharton and Tzagoloff (1967) and Rafael (1983). Percoll fractions containing mitochondria were assayed for total protein following the method of Bradford (1976) (Bio-Rad Laboratories, Richmond, California) using bovine γ-globulin (Sigma Chemical Co) as the reference protein. Cytochrome c (Sigma Chemical Co) was reduced with sodium dithionite and purified on a Sephadex G-25 column. The reduced cytochrome c was divided into 0.5-ml aliquots and stored at −80°C. The mitochondrial fractions were adjusted to a final protein concentration of 50 μg/ml. A sample (200 μl) was incubated with 75 μl Triton X-100 (2%, v/v), 1.5 ml KH_2PO_4 (40 mM, pH 6.7, containing 1 mM EDTA), 1.125 ml distilled water, and 100 μl cytochrome c (0.6 mM, reduced). The rate of disappearance of reduced cytochrome c was monitored at 550 nm on a Gilford Model 250 spectrophotometer (Gilford, Oberlin, Ohio). Potassium ferricyanide was added to the reaction mixture to completely oxidize the cytochrome c, and the absorbance was recorded.

Oxygen Consumption

Isolated mitochondria were suspended in homogenization buffer (0.225 M sucrose, 10 mM potassium phosphate, pH 7.4, 5 mM MgCl, 20 mM triethanolamine buffer, pH 7.4) and assayed for total protein. Oxygen consumption, respiration rates, and acceptor control ratios of isolated germ cell mitochondria were determined with a Clark oxygen electrode (Estabrook, 1967). Mitochondrial suspension was 0.33 g of protein for each assay. The final concentration of succinate was 160 mM, and the final concentration of ADP was 180 μM.

Mitochondrial DNA

Isolated mitochondria were resuspended in a 5 mM Tris-HCl buffer (pH 7.4) containing 0.225 M sucrose and 1 mM EGTA. Immediately after resuspension the mitochondria were diluted 1:1 with a 0.125 M Tris-HCl buffer containing 2% SDS, 2% 2-mercaptoethanol, and 50% glycerol and vortexed for 30 seconds (Guerin and Pelissier, 1992). A 10-μl aliquot was removed and bromophenol blue added, then the aliquot was loaded on a 1% agarose gel prepared in 1 × Tris-borate/EDTA (TBE) buffer.

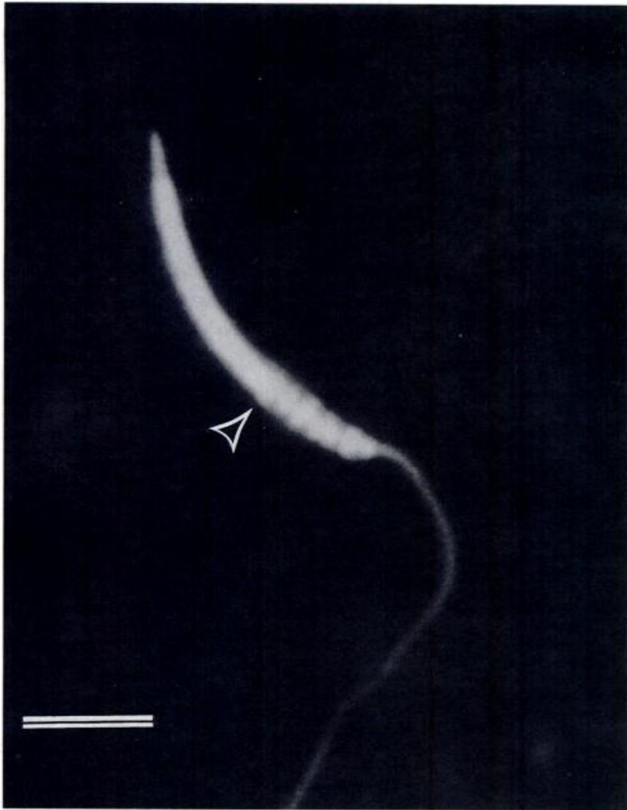

FIG. 1. Turkey spermatozoa stained with rhodamine 123 and acridine orange. Mitochondria can be visualized on the midpiece (arrow) due to the uptake of rhodamine 123 (bar = 25 μm).

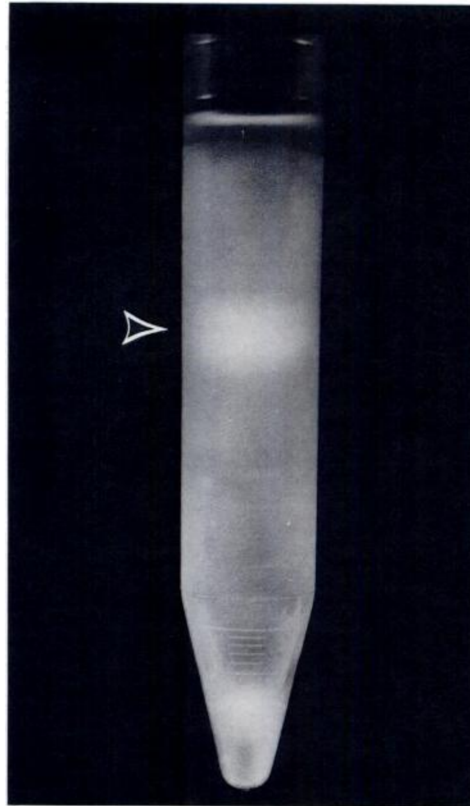

FIG. 2. Mitochondrial band (arrow) formed after centrifugation using a Percoll gradient.

The gel was electrophoresed for 1.5 hours at 5 mA/cm gel. The remaining solution was extracted twice, each with an equal volume of buffer-saturated phenol, an equal volume of a 1:1 mixture of phenol and chloroform: isoamyl alcohol (24:1), and an equal volume of chloroform: isoamyl alcohol (24:1). One-tenth volume of 3 M sodium acetate was added followed by 2.5 volumes of ice-cold ethanol (Maniatis et al, 1982). The sample was frozen overnight at −70°C and centrifuged for 1 hour at 12,000 × *g* and 5°C. The supernatant was removed, and the pellet was dried under a vacuum. The pellet was resuspended in TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0).

Restriction endonucleases and marker DNA were obtained from Gibco BRL, and digestion conditions were as recommended. Purified mtDNA and restriction enzyme digestions were electrophoresed on a 1% agarose gel prepared in 1 × Tris-acetate/EDTA (TAE) buffer for 2 hours at 7 mA/cm gel. Gels were stained with ethidium bromide and photographed using ultraviolet illumination with a Polaroid (Cambridge, Massachusetts) MP4 camera.

Results

A typical turkey sperm cell stained with rhodamine 123 and acridine orange is illustrated in Figure 1. The midpiece contained approximately 20–30 mitochondria (Thurston and Hess, 1987) that have taken up rhodamine 123, a reflection of their active oxidative state. Mechanical disruption, sonication, and centrifugation over Percoll was an effective procedure to isolate the mitochondria (Fig. 2). Using transmission electron microscopy, it was shown that the mitochondrial band removed from the final centrifugation of disrupted sperm over Percoll contained mostly mitochondria with no intact cells (Fig. 3). Higher magnification of tannic acid-fixed mitochondria showed that most mitochondria were in the orthodox state and the membranes were intact (Fig. 4). The mitochondria were elongate and ranged from 0.8 μm to 1.4 μm in length and approximately 0.25 μm in width.

The mitochondria appeared to be functionally intact as cytochrome c was oxidized at a rapid rate, indicating that the cytochrome c oxidase complex was capable of transferring electrons. The mitochondria also accumulated rhodamine 123, a fluorescent dye that is taken up by mitochondria when they are capable of generating a negative electrochemical gradient through activity of the proton pump (Johnson et al, 1980, 1981). The values of the ADP:O ratio and the respiratory control ratio (RCR) were 1.7 ± 0.313 and 4.6 ± 0.929, respectively ($N = 7$). These findings support the claim that the aforementioned pro-

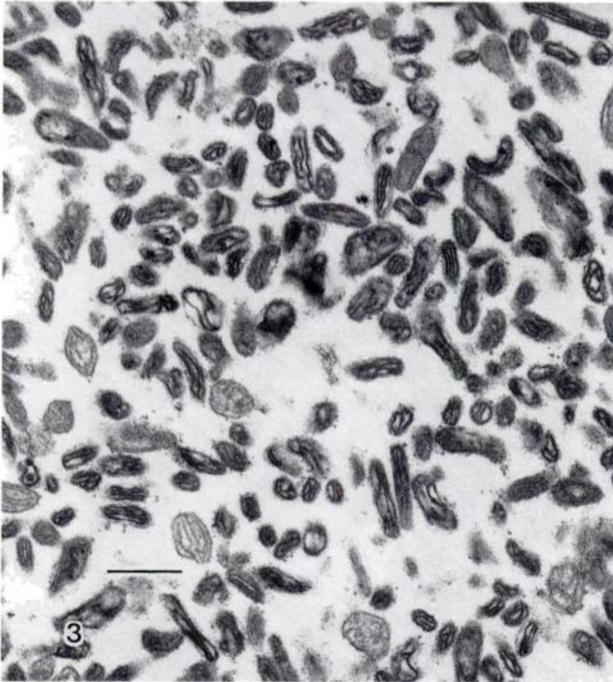

FIG. 3. Transmission electron micrograph of turkey spermatozoal mitochondria removed from Percoll showing purity and integrity of the mitochondrial fraction (bar = 1 μm).

cedure allows the isolation of morphologically and functionally intact mitochondria.

The purity of the mitochondria was sufficient to allow isolation of their DNA without the need of a cesium chloride gradient and long centrifugation times. The mtDNA and restriction endonuclease fragments were easily distinguished on agarose gels, and the restriction enzyme fragment pattern of mtDNA of liver and testis was identical to that of mtDNA purified from spermatozoa (Fig. 5a,b). The mitochondrial genome was found to be 16.457 ± 0.07 kbp (N = 9). Cutting the genome with EcoR1 produced four fragments, HindIII cut the genome in 3 places, and BamH1 cut the genome at one site.

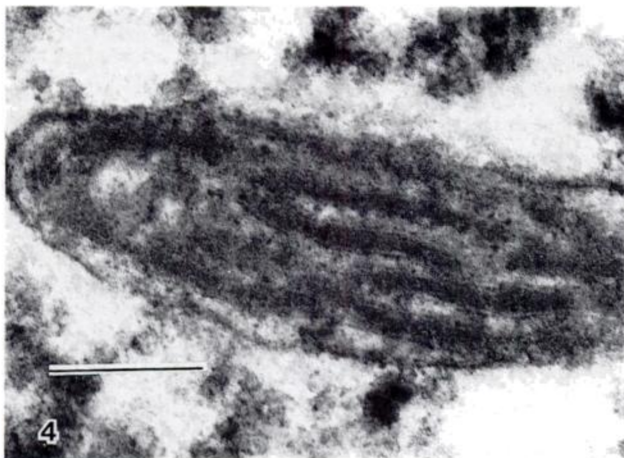

FIG. 4. High magnification of turkey spermatozoal mitochondria fixed with tannic acid (bar = 0.1 μm).

Discussion

The relative simplicity of spermatozoa reduces the need for detailed purification steps necessary with somatic cells to separate mitochondria from other organelles or debris. With the procedure outlined here, mitochondria can be readily obtained from spermatozoa and used for DNA or metabolic studies. However, it should be emphasized that spermatozoal mitochondria may have morphological, structural, and functional differences from somatic cell mitochondria (Fawcett, 1970; De Martino et al, 1979; Pallini, 1979; Hecht and Bradley, 1981).

Turkey spermatozoa contain 20–30 mitochondria along the midpiece (Thurston and Hess, 1987), whereas mammalian spermatozoa contain approximately 80 mitochondria (Hecht et al, 1984) on a longer midpiece. This and other differences between avian and mammalian mitochondria could be investigated because the isolation procedure described herein should work with a variety of different sperm types because the high lipid content of the organelle (Tzagoloff, 1982) makes it more buoyant than other sperm components. Therefore, the mitochondria form a band on top of dense gradients, whereas the other sperm components pellet.

Due to the condensed form of genomic sperm DNA, the sperm nuclei are resilient and dense and therefore are easily separated from the mitochondrial fraction. This results in an enriched mitochondria in which mtDNA could be isolated and cut with restriction endonucleases to determine the size of the mitochondrial genome. With minimal purification, restriction fragment patterns were demonstrated using EcoR1, HindIII, and BamH1 endonucleases.

The size of the mtDNA was found to be 16,457 ± 70 bp, which is very close to the reported sizes of other avian species (Shields and Helm-Bychowski, 1988; Desjardins and Morais, 1990). Glaus (1980) reported the size of turkey liver mtDNA to be 16,340 ± 230. The slight difference in the size may be due to the source of the mtDNA, as Glaus used the species *meleagris,* whereas the species *gallopavo* was used in this study. The restriction enzyme EcoR1 generated four fragments of the genome in this study, whereas Glaus (1980) reported that EcoR1 produced seven fragments in two turkeys and six in one turkey. This difference is most likely due to the different strain of turkey used. The restriction enzyme patterns with HindIII and BamH1 were the same.

The use of mtDNA in population genetic studies and research into mitochondrial-related diseases has made it

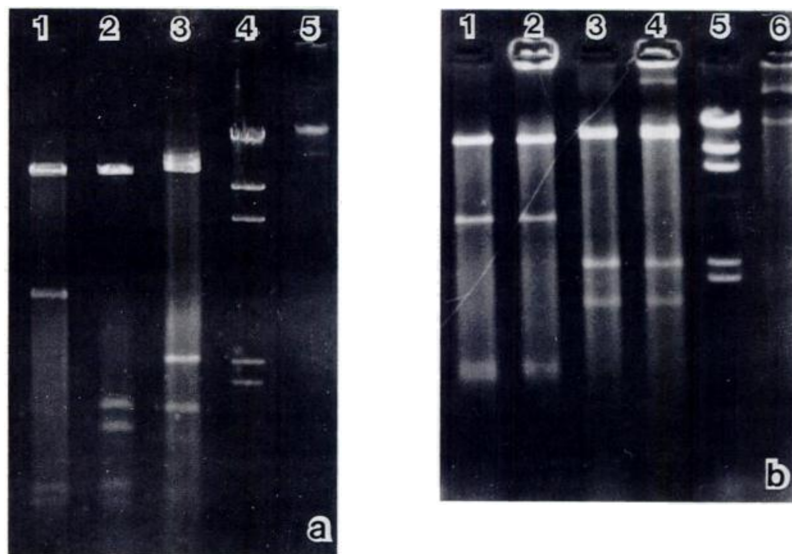

FIG. 5. (a) EcoRI (lane 1) and HindIII (lane 3) enzyme patterns of turkey spermatozoal mtDNA. Lane 2 is a double digest using EcoR1 and HindIII. Lane 4 contains HindIII digests of lambda phage DNA as molecular weight markers. Lane 5 is uncut mtDNA. EcoR1 generates four bands while HindIII generates three. **(b)** EcoR1 and HindIII enzyme patterns of liver and testis mtDNA (lanes 1 and 3) and spermatozoal mtDNA (lanes 2 and 4) showing identical restriction enzyme patterns. Lane 5 is molecular weight markers, and lane 6 is uncut spermatozoal mtDNA.

the most well-known piece of eukaryotic DNA (Avise, 1986; Wallace, 1992). It is widely believed that mtDNA is maternally inherited and that the occurrence of a paternal contribution of mtDNA is very low (Lansman et al, 1983). The possibility exists that mtDNA of the sperm is altered so that it can be selected against once it enters the oocyte. This possibility appears unlikely because in mice neither the oocyte nor the sperm mtDNA is methylated (Hecht et al, 1984). Also, it was shown that there was an 8–10-fold decrease in the number of mitochondrial genomes per haploid genome during spermiogenesis (Hecht et al, 1984), indicating that a selection against mtDNA may occur. Further research is needed to characterize differences between spermatozoal and somatic mtDNA and to determine why sperm mtDNA is not inherited.

In conclusion, the procedure described herein represents a rapid way to purify sperm mitochondria that are suitable for metabolic and nucleic acid research.

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