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Reconstruction and Explanation of Early Artifactual Microscopic Observations of Sperm Tail

M. Markova, V. Nikolova, L. Chakalova, S. Salieva**, Ts. Marinova****

Department of Biology, Medical Faculty, Medical University of Sofia
**Institute of Molecular Biology, Bulgarian Academy of Sciences, Sofia*
***Student at the Medical University of Sofia at the time of study*
****Department of Biology, Medical Genetics and Microbiology, Medical Faculty, Sofia University "St. Kliment Ohridski"*

Microtubules forming the sperm axoneme remain parallel until their termination in the terminal (end) piece of the tail. However, a bouquet-like arrangement of their distal ends was described by different microscopists in the 1950s and 1960s. We have reconstructed these results by detergent treatment of sperm cells prior to fixation, suggesting that the early artifactual observations may have been due to accidental detergent exposure. We suppose that extrapolation of the observed bouquet-like structure as a universal characteristic of mammalian sperm tail has been due to preference of higher complexity which may be regarded as a general bias in microscopic research. The present study is, to our knowledge, the first use of detergent treatment to reproduce and explain historical microscopic observations.

Key words: sperm, axoneme, microtubules, microscopy, artifact

Introduction

Scientific development depends not only on accumulation of new data but also on recognition and correction of erroneous findings. When this happens, exposed artifacts are in most cases abandoned as being of no interest to investigators. However, knowing the origin of inaccurate observations could be of factological and methodological value. For that reason, we have attempted to reproduce and explain some early sperm tail microscopic images now considered artifactual.

In one of the first studies of sperm cell ultrastructure, Bayle and Bessis [2] described in human spermatozoa 9-12 "fibrils" or "protofibrils" extending along the tail and forming its terminal (end) piece. According to the authors, protofibrils or "terminal filaments" (i.e. microtubule doublets) in this last tail domain were sometimes grouped together, but in most cases were arranged as a bouquet which was shown in three of the seven figures in the article. One of them, Fig. 7, was a whole-mount electron micrograph specifically devoted to the "filaments" in the terminal piece.

It seems that most microscopists studying the axoneme quickly noted the artifactual nature of bouquet-like "terminal filaments", while accepting other findings described in the same work. Afzelius [1] cited [2], and more precisely Fig. 7, not as showing bouquet-like end piece but as evidence that the outer "filaments" are double and may appear forked in their distal ends – a peculiarity which was later confirmed. As electron microscopy techniques improved, early artifacts were recognized and cleared. In Fawcett's landmark 1975 review *The mammalian spermatozoon* [6], the end piece was described as formed by axoneme and overlying cell membrane and no controversy over its structure was mentioned. This concept is still valid today, with new research only confirming the conservatism and universality of axonemal structure across the mammalian class [7] and beyond it [9].

In 1965, Bulgarian researcher of animal reproduction Kiril Bratanov wrote that "central fibril" (i.e. axoneme) in the end of the tail, if observed by electron microscope, shows branching to 9-12 "tail filaments" with greater length than the tail itself [15]. The figure illustrating this remark (Fig. 61) was a reproduction, with appropriate credit, of Fig. 7 from [2]. More intriguingly, another illustration (Fig. 63) showed drawings of ungulate sperm cells, each presented with a bouquet of 9 filaments at the end of its tail. No credit was given for this image, implying that it had been drawn by the author based on his own observations. The concept of "tail filaments" radiating from the terminal piece is still remembered at Bratanov's institute, though it has not been studied further and is now considered outdated [16].

As far as we know, no attempts have been made to elucidate the factors causing bouquet-like appearance of sperm tail. We presumed that it might have been mediated by membrane damage. To test this hypothesis, we sought to inflict deliberate membrane damage in order to monitor its effect on sperm tail morphology. We treated sperm cells with detergent, observed them by light and electron microscopy and compared the results to the above cited early reports.

Materials and Methods

Human ejaculated spermatozoa were obtained from the In Vitro Fertilization Laboratory, Department of Biology, Medical University of Sofia. Mouse spermatozoa were obtained from the vas deferens as described in [13].

After washing with phosphate-buffered saline (PBS), pH 7.2, the cells were extracted for 10 min at 4°C with 0.5% Triton X-100 in PBS with 1.2 mM phenylmethylsulfonylfluoride. Then they were washed twice with PBS and prepared for microscopy. Sperm cells not treated with Triton X-100 were processed parallelly as controls.

For whole-mount electron microscopy, washed spermatozoa were left to adhere to formvar-coated grids. Then the cells were fixed with 2.5% glutaraldehyde in PBS for 30 min at 4°C and washed again with PBS. Postfixation was performed for 5 min with 1% OsO_4 in PBS at 4°C. The cells were washed twice with water and negatively stained for 2 min with 1% uranyl acetate.

Immunofluorescence was carried out according to [14]. Spermatozoa were dropped onto slides, left to air-dry and fixed with methanol for 5 min and acetone for 2 min at 4°C. After rehydration, they were treated for 1 h at room temperature with anti-alpha tubulin monoclonal antibody TU-01 (Institute of Molecular Genetics, Prague, described in [3] and [10]) and then for 30 min with FITC-conjugated anti-mouse polyvalent Ig (Sigma). PBS with 1% bovine serum albumin was used for rehydration, antibody dilution and washing. In negative controls, anti-tubulin antibody solution was replaced with dilution buffer alone. After that, slides were mounted for immunofluorescence in a mixture of PBS and glycerol 1:9 (v/v) with 2.3% (w/v) DABCO (Sigma).

For conventional light microscopy, cells were dropped onto slides and fixed with methanol for 5 min. After air-drying, they were stained with modified Giemsa (Sigma) according to the manufacturer's instructions. The 0.4% buffered stock solution was diluted 1:20 with distilled water and applied for 15 min. Then the slides were rinsed with distilled water and left to air-dry before observation.

Results

Human and mouse sperm cells treated with Triton X-100 showed similar bouquet-like terminal pieces by all three methods of microscopic observation. Whole-mount electron microscopy showed microtubule doublets radiating caudally from the fibrous sheath (Fig. 1A) in a way reminiscent of the images in [2] (Fig. 1B).
At light microscopic level, the photomicrographs of extracted spermatozoa (Fig. 2A, B) were similar to the drawings in [15] (Fig. 2C). Immunofluorescent staining for alpha-tubulin clearly visualized the "bouquets" in the end piece (Fig. 2A). In Giemsa-stained cells, end pieces were less clear and did not allow visualization of individual microtubules and doublets. However, the general appearance of the "bouquet" was detectable in a substantial number of cells, although the images were difficult to observe, document and interpret (Fig. 2B). Unextracted sperm cells showed unbranched, thread-like terminal pieces (data not shown).

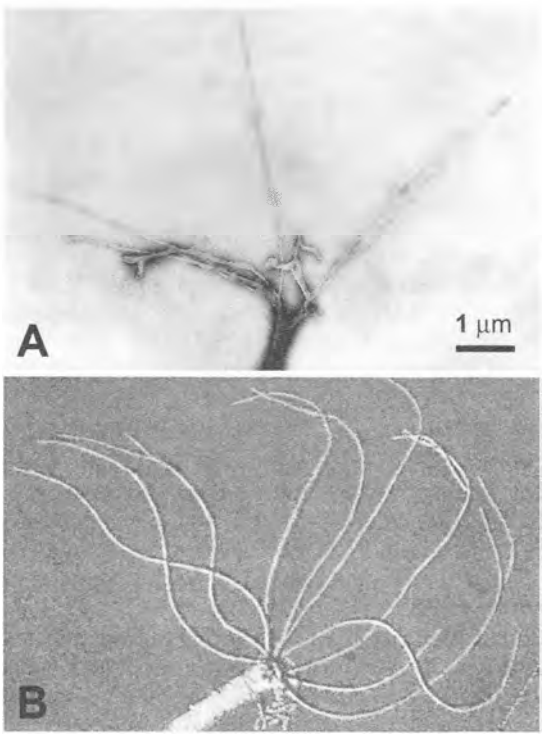

Fig. 1. Whole-mount electron micrographs of human sperm tail bouquet-like end pieces. **A**. A cell treated with Triton X-100 before fixation. **B**. For comparison, Fig. 7 from [2], reproduced with publisher's permission.

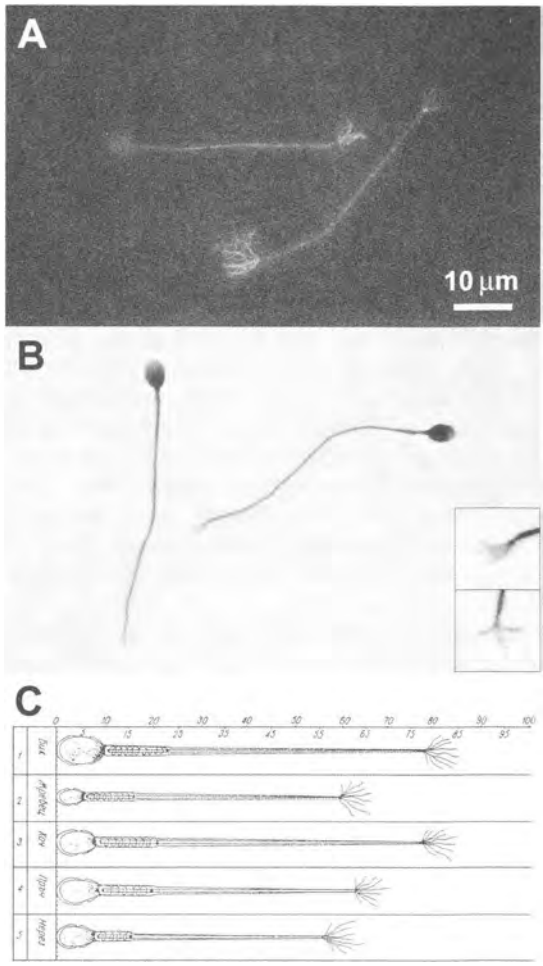

Fig. 2. Light microscopic images of spermatozoa showing bouquet-like end pieces. **A**. Immunofluorescence for alpha-tubulin in human sperm cells pretreated with Triton X-100. **B**. Giemsa-stained human sperm cells pretreated with Triton X-100, at the same magnification. Tail terminal pieces are shown at right as magnified insets. **C**. For comparison, Fig. 63 from [15], reproduced with publisher's permission and showing spermatozoa of farm animals (from top: bull, stallion, ram, buck and boar) drawn to the same scale to compare their sizes.

Discussion

As we know today, axonemal doublets *in vivo* are strictly parallel and remain close to each other until their microtubules terminate near the end of the tail. So far, there has been no explanation for the artifactual bouquet-like terminal pieces published in the early reports. Mammalian sperm cytoskeleton is very sensitive to the procedures used to prepare cells for microscopy [5]. It should be noted that, while in more proximal

tail domains the axoneme is encircled by mitochondrial and fibrous sheath, in the end piece it is overlaid only by the cell membrane. If the latter is damaged, nothing prevents microtubules from dispersing in a bouquet-like pattern. We treated spermatozoa with Triton X-100 in order to introduce this effect deliberately. In our earlier studies, we have used the same detergent to reveal cytoskeletal association of cell surface receptors [11] or cytoskeletal nature of little-known structures [12]. The present study is, to our knowledge, the first use of detergent treatment to reproduce and explain historical microscopic observations.

We find it probable that bouquet-like end pieces in the early reports [2, 15] resulted from unreported detergent exposure. It is unlikely that membrane-damaging ingredients have been used on purpose in these studies, but accidental contamination could easily occur, e.g. from detergent-washed and not properly rinsed glassware. An additional problem concerning [15] was how the author could make his observations, after he, to the best of our knowledge, had no access to electron microscope, immunofluorescence equipment or the combination of phase-contrast microscope and shadowing used in [2] to visualize the "bouquet" at light microscopic level. In an attempt to reproduce his work, we stained detergent-treated spermatozoa with Giemsa, a very popular dye for cell smears. The results showed that this approach, while much less informative than electron microscopy or immunofluorescence, still allowed observation of the terminal piece "bouquets", although the diameter of individual doublets was below the light microscope resolution.

Our successful reproduction of the artifactual bouquet arrangement still does not explain how it could be observed in most (or all) cells in the early studies. We find it likely that the authors, seeing two alternative appearances of the terminal piece in different sperm tails, assumed that the variation showing more details reflects the real structure. In other words, there may have been bias in favour of higher complexity. The same phenomenon may have contributed to the perception of other artifactual structures as real, e.g. the microtrabeculae, thought in the late 20th century to constitute a fourth cytoskeletal system [8], and the bacterial mesosome [4]. Our opinion is that complexity bias in microscopic research should be kept in mind, although the enormous real complexity of living structures makes it very difficult to avoid.

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