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RESEARCH ARTICLE

Fine Structure of Zebrafish (*Danio rerio*) Spermatozoa

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ABSTRACT

The microstructural and ultrastructural examination of zebrafish (*Danio rerio*) spermatozoa was performed to detect the structure, geometrical feature and morphological parameters. Milt samples from 30 zebrafish were examined by use of phase contrast microscopy (PCM) and transmission electron microscopy (TEM). The zebrafish spermatozoa measured 32.79±1.97 μm in total length and had a non-acrosomal spherical head, a short cone-shaped midpiece and a flagellum. Heads were lopsided and almost occupied by nucleus with homogeneous dense chromatin, except for a few small electro-lucent vacuoles. The shallow nuclear fossa was mid-laterally located on the nuclear surface and contained the proximal centriole, which was tilted to one side of nucleus and placed at a 125º angle against distal centriole. In the midpiece, the cytoplasmic sheath showed asymmetric organization with round or bended mitochondria gathered at one side, while at the thinnest place, was only little cytoplasm. The flagellum, 29.32±1.98 μm in length and 242.56±19.27 nm in width, was eccentrically positioned in relation to the nucleus. It had a "9×2 + 2" pattern axoneme, however, there was lack of central microtubules at transition between flagellum and distal centriole. In addition, a few vesicles appeared between the axoneme and the cytoplasmic membrane. Based on the observations, the schematic organization of zebrafish spermatozoon was drawn. The zebrafish spermatozoa possessed some structural features that were specific to cyprinid family, however, they exhibited interspecific differences.

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INTRODUCTION

The zebrafish *Danio rerio* (Cyprinidae, Teleostei) is a common aquarium freshwater fish, which has become a popular model specie suited for studies of genetics (Norton and Bally-Cuif, 2010), toxicology (Sipes *et al.*, 2011) and diseases (Ali *et al.*, 2011). They have been frequently used in the fields of veterinary sciences in recent years, such as an infection model for *Streptococcus suis* type 2 (Jun-yi *et al.*, 2007) and for evaluating the toxicity of veterinary pharmaceuticals (Carlsson *et al.*, 2013). The germ cells in zebrafish have been applied to toxicological study as well (McAllister and Kime, 2003; Thresher *et al.*, 2011). As a model organism, more morphological, biochemical and physiological information is required for normal zebrafish.

The teleost spermatozoa exhibit a wide range of structural variations among species that could be valuable in evolutionary, taxonomic and phylogenetic research (Mattei, 1991; Fürböck *et al.*, 2009). The morphology of spermatozoa has been studied in many cyprinid species, such as tench *Tinca tinca* (Psenicka *et al.*, 2006; Pšenička *et al.*, 2010), *Barbus barbus* (Alavi *et al.*, 2008), *Chondrostoma nasus* (Fürböck *et al.*, 2010), and *Tribolodon hakonensi* (Neznanova, 2012), whereas information on that of zebrafish spermatozoa remains limited.

The present study thus aimed to obtain detailed information of zebrafish spermatozoa, employing both phase contrast microscopy (PCM) and transmission electron microscopy (TEM). This work could contribute

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in knowledge about reproductive biology of the zebrafish as tools for phylogenetic research, as well as providing a normal morphological data for evaluation of sperm quality in sperm cryopreservation and toxicological studies.

MATERIALS AND METHODS

Sexually mature *D. rerio* males were reared under controlled photoperiod (daily range: 14 h:10 h LD; temperature, 28-30°C and pH, 7.0-7.8) with recirculation aquaculture system at Shanghai laboratory animal research center. A total number of 30 *D. rerio* males, separated from females for seven days, were anesthetized with MS222 (90 mg/l) on the same day. About 3µl milt was collected from each male. It was then pooled and fixed in 2.5% glutaraldehyde in phosphate buffered saline (PBS; 4ºC, pH 7.4, 0.1 M) for 1h. For PCM, samples were dropped on glass slides and examined under an Olympus BX53 microscope. For TEM, samples were concentrated by centrifugation and then postfixed in 1% osmium tetroxide in the same buffer for 1 h. After double fixation, samples were dehydrated and embedded in Epon 812. The blocks were ultrathin sectioned (50 nm), and then these sections were mounted on Formvar-coated grids, stained with uranyl acetate and lead citrate and examined in a HITACHI H-7650 transmission electron microscope. The morphometric parameters were measured from at least 30 randomly chosen spermatozoa.

RESULTS

The spermatozoon of zebrafish had a total length of 32.79±1.97 µm (in the 24.56-37.49 µm range), were shaped like a golf club and differentiated into a lopsided non-acrosomal spherical head, a short cone-shaped midpiece and an eccentric tail (Figs. 1a-b, 2a). According to these observations, the schematic organization of spermatozoa and corresponding transversal sections are shown in Fig. 4.

As seen in ultrathin sections with TEM, the heads were occupied almost by nucleus, which contained homogeneous dense chromatin, except for some small electron-lucent vacuoles (Figs. 2c, 4a). A shallow nuclear fossa, arising from inward depression of nuclear membrane, was mid-laterally situated on nucleus surface, containing part of centrioles (Figs. 2b, 4b-c). The proximal centriole was tilted to one side of nucleus (Fig. 4b) and arranged at an angle of 125º against distal centriole (Fig. 2b). The distal centriole was almost tangent to the nucleus and acted as a basal body.

The midpiece was connected with the posterior end of nucleus. The cytoplasmic membrane invagination between the flagellum and the cytoplasm created a channel (Figs. 3a-b), which was narrow and deep, ending with the terminal of distal centriole (Figs. 2c, 3a-b). The cytoplasm around the cytoplasmic channel formed cytoplasmic sheath and surrounded the initial segment of flagellum. The cytoplasmic sheath was asymmetric, thick at one side of flagellum while thin at the opposite side, left only a little cytoplasm at the thinnest part (Figs. 3b, 4f). The cytoplasmic sheath housed 2-6 round or bended mitochondria, having 270.27±49.36 nm length in minor axis (205.7-378.9 nm range), concentrated at one side

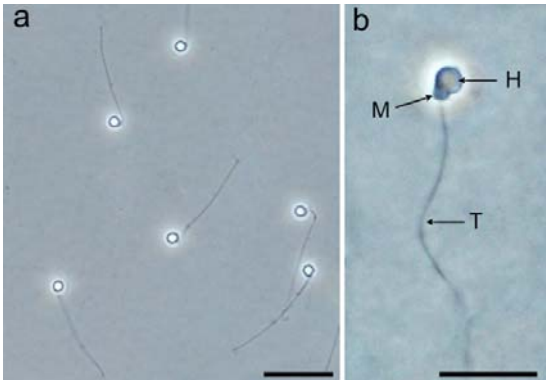

Fig. 1: Zebrafish spermatozoa (PCM) showing (a) Spermatozoa in the shape of a golf club, (b) Spermatozoon consisted of a spherical head (H), a midpiece (M) and a tail (T). Scale bar: a: 20µm; b: 10 µm.

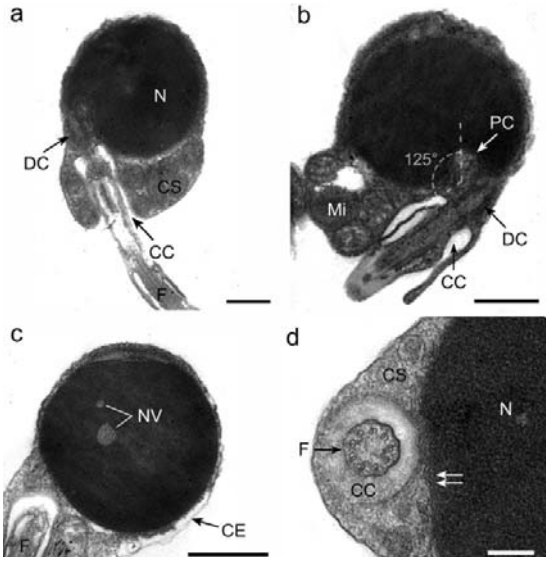

Fig. 2: Zebrafish spermatozoa (TEM) showing: (a) Longitudinal section through centrioles and flagellum, (b) The proximal centriole arranged at an angle about 125° against the distal centriole, (c) Head with nuclear vacuoles and (d) Axoneme without central microtubules between distal centriole and flagellum. CC: cytoplasmic channel; CE: cytoplasmic membrane; CS: cytoplasmic sheath; double arrow: nuclear membrane; DC: distal centriole; F: flagellum; N: Nucleus; NV, nuclear vacuoles; Mi: mitochondria; PC: proximal centriole. Scale bar: c:1µm; a, b:600nm; d: 200nm.

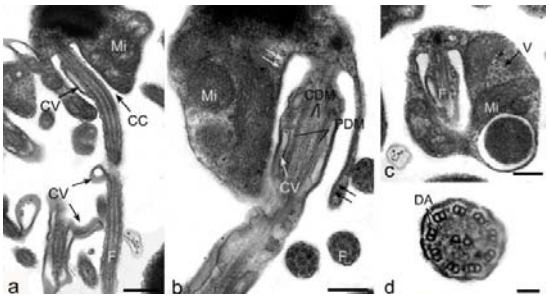

Fig. 3: Midpiece and flagellum of zebrafish spermatozoa (TEM) showing: (a) Midpiece with few mitochondria in asymmetric cytoplasmic sheath, (b) The cytoplasmic sheath with double layer inner membrane (arrow), and the bended mitochondria at one side, (c) A small amount of vesicles between mitochondria, (d) Axoneme in 9×2 + 2 pattern with both dynein arms. CC: cytoplasmic channel; CDM: central doublet of microtubules; CV: cytoplasmic vesicles; DA: dynein arms; double arrow: double layer membrane; F: flagellum; Mi: mitochondria; PDM: peripheral doublets of microtubules; V: vesicles. Scale bar: a: 600nm; b: 300nm; c: 400nm; d: 60nm.

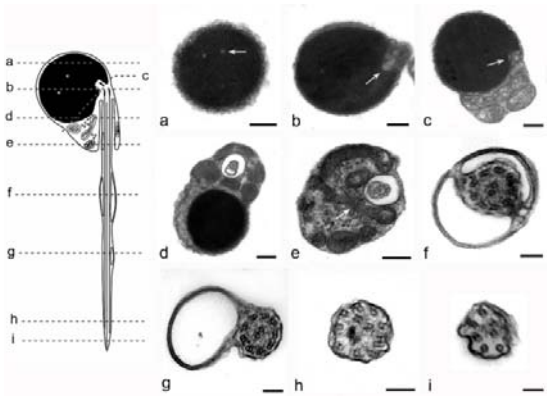

Fig. 4: Schematic organization of zebrafish spermatozoa (left) and corresponding cross section with TEM (right) showing: (a) small nuclear vacuoles in nucleus (arrow), (b) The lopsided proximal centriole (arrow), (c) Cross section through proximal centriole (arrow), (d) Cytoplasmic sheath and flagellum laterally located, (e) The bended mitochondria (arrow) at one side, (f-g) Flagellum with vesicles and (h-i) The distal flagellum with scattered dispersing singlets microtubules. Scale bar: a-e: 500 nm; f-h: 100nm; i: 60nm.

(Figs. 3a-c, 4d-e). The membrane of the inner cytoplasmic sheath presented a double layer membrane (Fig. 3b). Besides, there were small amounts of moderate electron dense vesicles between mitochondria (Fig. 3c).

The flagellum, 29.32±1.98 μm long (in the 22.58-34.45 μm range) and 242.56±19.27 nm wide (in the 192.2-298.0 nm range), consisting of the axoneme enclosed by the plasma membrane (Fig. 3d). Several big and small cytoplasmic vesicles between the axoneme and the cytoplasmic membrane emerged at one side of flagellum or encircling the axoneme (Figs. 3a-b, 4f, g). The axoneme had a width of 192.48±12.67 nm (in the 170.11-211.05 nm range), showing a typical "9×2 + 2" pattern with two dynein arms and without intratubular differentiations (Fig. 3d). While at transition between flagellum and distal centriole, there was absent of central microtubules in "9×2 + 0" pattern (Fig. 2c). At the end of flagellum, doublets dispersed into singlet microtubules (Fig. 4h), and gradually reduced in number to six or even less (Fig. 4i).

DISCUSSION

The absence of acrosome is a common feature of the spermatozoa in fishes with external fertilization (Mattei, 1991), excepted for few species, such as sturgeons (Psenicka *et al.*, 2007). Correspondingly, the micropyle is present on the egg for penetration of spermatozoon (Amanze and Iyengar, 1990). The configuration of zebrafish spermatozoa were similar to those of aquasperms of external fertilizing fishes (Mattei, 1991). However, in this study they were found sharing some characteristics with cyprinid spermatozoa, and exhibited interspecific differences with them.

This study demonstrated that the zebrafish spermatozoa had an asymmetric shape because of the lateral location of nuclear fossa, leading to the head being lopsided and the flagellum inserted eccentrically. The ancestral spermatozoa of the Neopterygii possess a concavity at the base of rounded nucleus where the centrioles are situated (Mattei, 1991). Different from ancestral mode of Neopterygii, the concavity of cyprinid and esocid spermatozoa was lateral (Mattei, 1991; Fürböck *et al.*, 2009), as in *Rutilus meidingerii* spermatozoon (Fürböck *et al.*, 2010), which emerged as nuclear fossa on the caudo-lateral region of the nucleus. In the present study, the nuclear fossa was detected being located medio-laterally on the nucleus. Furthermore, centrioles were positioned at 125° angle to each other. It has been observed that the diverse arrangements of centrioles existed in cyprinid spermatozoa, e.g. perpendicular in *Labeo victorianus* (Rutaisire *et al.*, 2006), 110° in *Rutilus meidingeii*, 125° in *C. nasus* (Fürböck *et al.*, 2010) and 140° tench in *T. tinca* (Psenicka *et al.*, 2006). This geometric diversity of spermatozoa structure may lead to different beating pattern of the flagellum among species, and affect the movement of spermatozoa during fertilization.

The chromatin in the nucleus of teleostean spermatozoa showed diverse condensed states. In *Brycon*, chromatin appeared as filamentous clusters, while in *Salminus*, the chromatin occured as thick fibres (Veríssimo-Silveira *et al.*, 2006). In zebrafish, the chromatin was detected slightly granular with few electron-lucent vacuoles, which is consistent with some cyprinid species as *B. barbus* (Alavi *et al.*, 2008) and *C. nasus* (Psenicka *et al.*, 2006), as well as in species of other families, e.g. *Gadus morhu* (Rebours and Ottesen, 2013) and *Lagodon rhomboids* (Gwo *et al.*, 2005). Chen (2000) suggested that nuclear vacuoles of spermatozoa probably functioned as reducing mechanical stress during vigorous movements at the time of fertilization. In contrast, Fürböck *et al.* (2009) reported no vacuoles existed in four subfamilies of Cyprinidae, which implied that the nuclear vacuoles of spermatozoa had a real difference among cyprinid species.

Unlike spermatozoa, species with external fertilization having a developed midpiece, as spermatozoa of American black bears with spiral mitochondria around the axoneme (Brito *et al.*, 2010) and turtle spermatozoa with spherical mitochondria containing concentric laminated membranes (Hess *et al.*, 1991; Bian *et al.*, 2013), the gametes of fishes with external fertilization have an underdeveloped midpiece. As seen in this study, only a few mitochondria lied in the cytoplasmic sheath of zebrafish spermatozoa. In cyprinid spermatozoa, two kinds of mitochondrial arrangement, concentrated at one side and irregularly distributed, have been reported by Fürböck *et al.* (2009). The distribution of mitochondria in zebrafish spermatozoa could be regarded as the latter mode.

The primitive spermatozoa of teleosts evolved along different lines, forming diverse flagellar apparatus, as biflagellate in *Cichlasoma dimerus* (Vázquez *et al.*, 2012), "9 + 0" structure of axoneme without central microtubules and external arms in *Conger myriaster* (Okamura and Motonobu, 1999), and the intratubular differentiations in *Spratelloides gracilis* (Gwo *et al.*, 2006). The zebrafish spermatozoa demonstrated "9×2 + 2" pattern of axoneme with both dynein arms, while at the transition between flagellum and centrioles, there was absence of central microtubules as other cyprinid species (Fürböck *et al.*, 2009). In addition, no lateral fins or ridges were found on the flagellum, while cytoplasmic vesicles were as frequently observed as in some cyprinid fishes, such as *T.*

tinca (Psenicka *et al*., 2006), *Labeo victorianus* (Rutaisire *et al*., 2006) and *C. nasus* (Fürböck *et al*., 2010).

Conclusion: The organization of zebrafish spermatozoa has comparable similarities with that of Cyprinidae. However, position of nuclear fossa, angle between centrioles, distribution of mitochondria and the presence or absence of nuclear vacuoles exhibits species-specific differences.

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