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An Ultrastructural Study on the Occurrence of Aberrant Spermatids in the Testis of the River Sculpin, *Cottus hangiongensis*

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Abstract The process of spermatogenesis and spermiogenesis in the river sculpin, *Cottus hangiongensis*, was observed ultrastructurally. During spermatogenesis, some germinal cysts in the seminal lobules were found to contain spermatocytes, which were provided with irregularly shaped nuclei, doughnut-shaped mitochondria, and atypical intercellular bridges with multiple disk-like cisternae. In addition, many cysts containing binuclear spermatids were observed in the testis. Within the condensed chromatin of the paired nuclei of the aberrant spermatids, highly electron-dense granules occurred, becoming the core of successively developing chromatin globules. The chromatin globules increased in size, resulting in an enlargement of the paired nuclei. These cells were finally released from the cyst into the lumen of the seminal lobules and underwent further degeneration, thus appearing as characteristic 'spermatid masses' in the mature testes.

The river sculpin, *Cottus hangiongensis*, is commonly found in the rivers of southern Hokkaido, Japan (Sato and Kobayashi, 1951; Goto, 1981). In a previous study, we reported that germinal cysts containing aberrant spermatids occurred in the testis and that these resulted in the formation of 'spermatid masses' during spermiogenesis in this cottid species (Quinitio et al., 1988). Hann (1927) was the first to report the occurrence of spermatid masses, in the testis of *Cottus bairdii*. Later, he observed spermatid masses in several species of freshwater and marine cottids (Hann, 1930). However, these studies were conducted only by light microscopy.

The present study deals with ultrastructural observations on the development of aberrant spermatids and spermatid masses, which resulted from incomplete cytoplasmic division of spermatocytes followed by nuclear degeneration, in the testis of *C. hangiongensis*. For comparison, brief descriptions are also given on the ultrastructural characteristics of normal spermatids and spermatozoa of this cottid species.

Materials and Methods

During their annual reproductive cycle in 1986–1987, adult males of the river sculpin, *Cottus hangiongensis*, were collected every month using a dip net in the lower reaches of the Tobetsu River, near Hakodate, Japan.

After anesthetizing the fish, the testes were dissected out and pieces fixed immediately in Karnovsky's 4% paraformaldehyde-2.5% glutaraldehyde mixture in 0.1 M cacodylate buffer (pH 7.4) for 2–5 h at room temperature, washed overnight in the same buffer, and then post-fixed in 1% osmium tetroxide in the same buffer (pH 7.4). Samples were embedded in epoxy resin following standard procedures. Ultrathin sections, cut on a Sorvall Porter-Blum MT-1 or Reichert-Nissei Ultracut N ultramicrotome with glass knives, were stained with uranyl acetate and lead citrate. Observations were done with a Hitachi H-300 or H-7000 electron microscope. Semithin sections were also cut and stained with methylene blue for light microscopy.

For scanning electron microscope observations, semen was extracted from the testis by making a small cut at the common sperm duct and then stripping the sperm reservoir toward its posterior end. A small volume of semen diluted with teleost Ringer's solution was then placed on a small piece of cover slip, which had been previously ion-sputtered and coated with 1% poly-L-lysine (Sigma Chem. Co., USA), and was fixed immediately with an equal volume of 2% glutaraldehyde for 1 h at room temperature. After dehydration in an alcohol series, the cover slips with semen were placed in isoamyl ace-

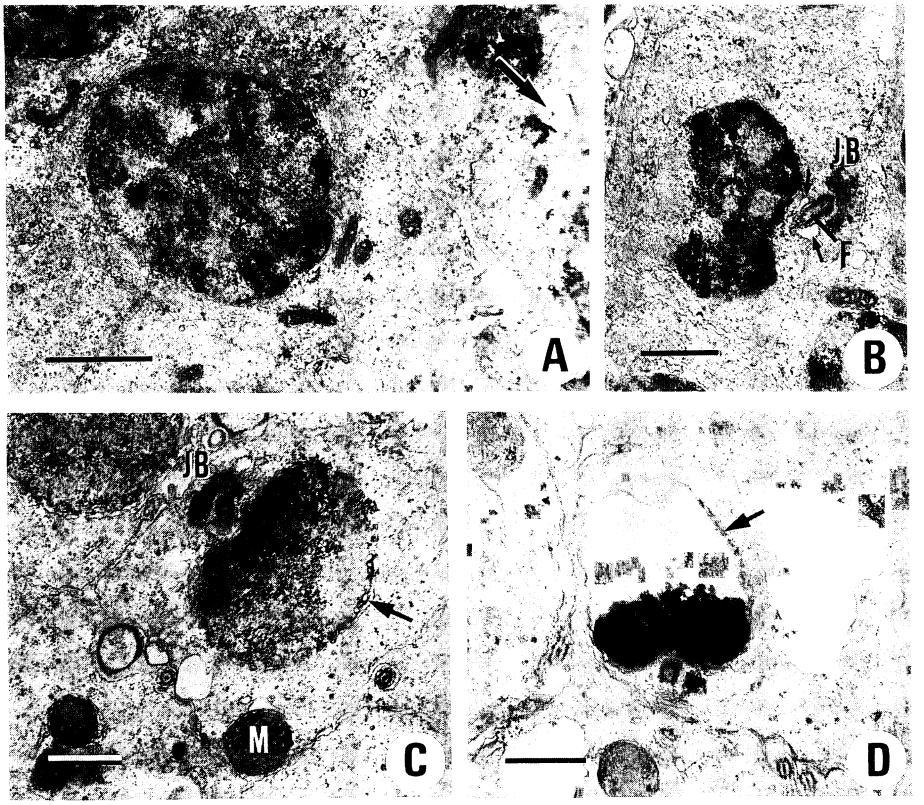

Fig. 1. Electron micrographs of a spermatocyte and spermatids of *Cottus hangiongensis*. F, flagellum; JB, juxtanuclear body; M, mitochondrion. A) A spermatocyte at the pachytene stage. Arrow shows an intercellular bridge connecting two adjacent cells. Scale bar, 2 μm. B) An early spermatid with characteristically dispersed chromatin and developing flagellum. Arrows indicate the cytoplasmic canal. Scale bar, 1 μm. C) An early spermatid showing breakages (arrow) along the nuclear membrane. Scale bar, 1 μm. D) A late spermatid showing nuclear membrane folding with bead-like structures (arrow) along the nuclear membrane. Scale bar, 1 μm.

tate, dried in carbon dioxide in a JEOL JCPD-3 critical point drier, mounted on stubs, coated with a thin layer of gold using a JEOL JFC-1100 ion sputter, and observed with a JEOL JSM-25 or Hitachi S-2300 scanning electron microscope.

Results

Normal spermatogenesis. Pachytene spermatocytes were characterized by a complex nuclear structure with the formation of clumps of chromatin and synaptonemal complexes (Fig. 1A). Numerous mitochondria, mostly elongated, were usually grouped at one pole of the cell. Each cell usually possessed several intercellular bridges lined by a typical thickening of the plasma membrane.

Early spermatids grouped in cysts were connected with each other by intercellular bridges (Fig. 1B–D). The nucleus was round to oval in shape, ranging from 2.8 to 3.2 μm in diameter. The chromatin in the nucleus was interspersed with less electron-dense areas. Some elongated mitochondria were seen in the cytoplasm. A large mass of coarsely granular material, which probably corresponded to the juxtanuclear body, lay next to the centrioles. A cytoplasmic canal, which separated the flagellum from the cell body, had developed. At this phase of spermatogenesis, the cells had a nuclear diameter of about 2.8–3.5 μm, and possessed two spherical mitochondria which seemed to move closer to the centriolar complex in the cytoplasm. As nuclear chromatin started to condense, breakages appeared on the nuclear membrane

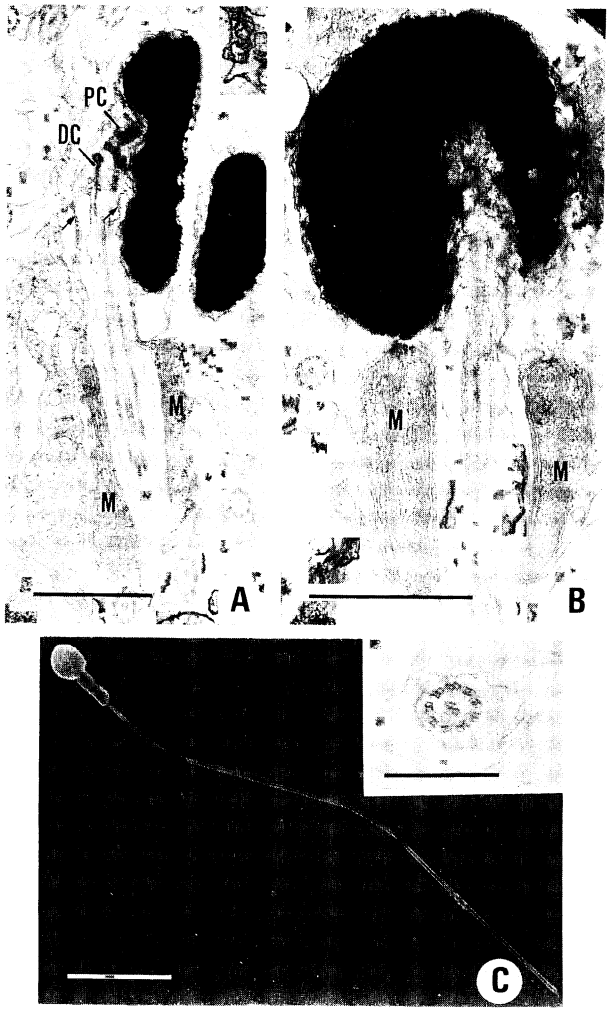

Fig. 2. Electron micrographs of spermatozoa of *Cottus hangiongensis*. DC, distal centriole; M, mitochondrion; PC, proximal centriole. A) A 'sagittal' section of a spermatozoon. Arrows indicate the point of reflection of the plasma membrane invagination. Scale bar, 1 μm. B) A 'frontal' section of the flattened sperm head. Two large lobes of the fused mitochondrion lie on the cytoplasmic canal. Scale bar, 1 μm. C) Scanning electron micrograph of a spermatozoon. Scale bar, 5 μm. Inset: a cross section of the sperm flagellum, showing the ring of doublet axonemal tubules. Scale bar, 0.5 μm.

at the side opposite the electron-lucent nucleoplasm. After the chromatin had condensed into a homogeneous disk-shaped mass, the greater portion of the nucleus was considerably electron-lucent, with the nuclear membrane again continuous. At a later phase, breakage again occurred along the electron-lucent side of the nuclear membrane, with the membrane starting to fold and form a large vacuole within the cytoplasm (Fig. 1D). This folding process continued until the nuclear membrane was in close contact with the condensed chromatin. In the late spermatid stages, Golgi apparatus and the juxtanuclear body were not observed in the cytoplasm.

Spermatozoon. The spermatozoon of the river sculpin consisted of a head devoid of an acrosome, a large midpiece, and a long flagellum (Fig. 2A–C). The nucleus was almost round and disk-like in shape measuring about 2.2–2.3 μm in diameter and 0.6 μm in depth at the thickest portion. The articular fossa extended from the caudal pole until about 3/4 of the

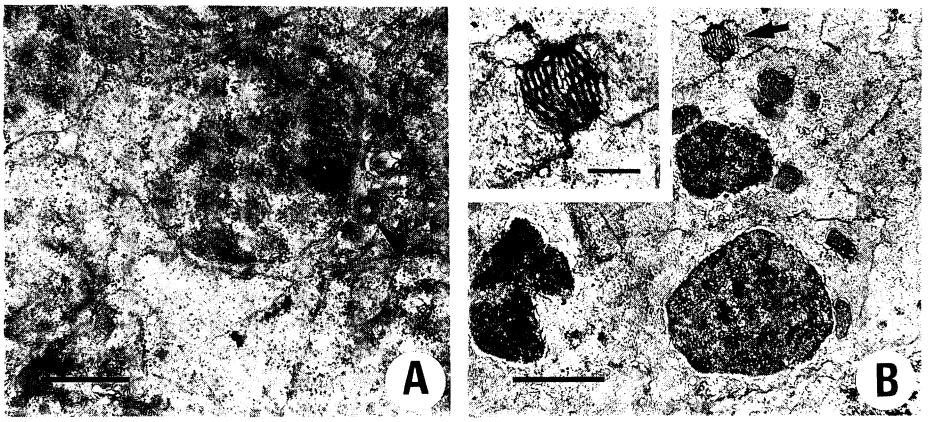

Fig. 3. Electron micrographs of atypical spermatocytes found in the testis of *Cottus hangiongensis*. A) A spermatocyte with prominent, doughnut-shaped mitochondrion (arrowhead). Scale bar, 2 μm. B) Spermatocytes having irregularly shaped nucleus and intercellular bridge with multiple disk-like cisternae (arrow). Scale bar, 2 μm. Inset: an enlarged view of the atypical intercellular bridge. Scale bar, 0.5 μm.

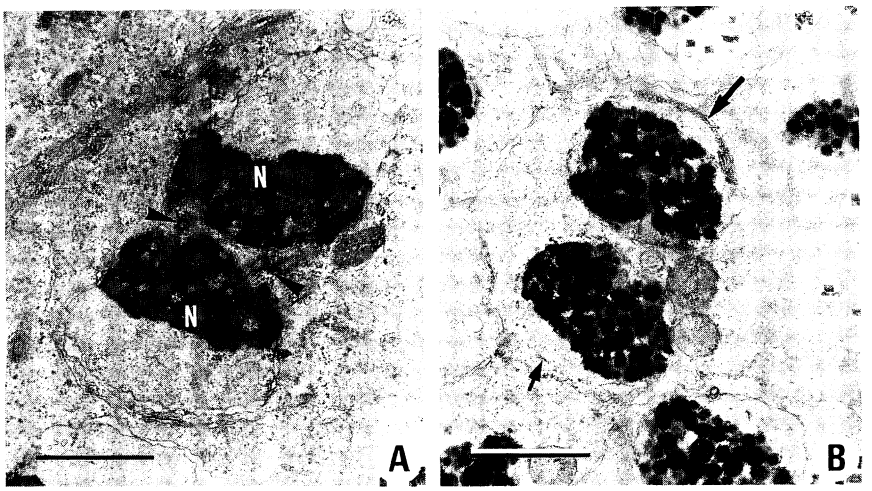

Fig. 4. Electron micrographs of different stages of aberrant spermatids of *Cottus hangiongensis*. N, nucleus. A) An early stage showing the condensed chromatin having electron-lucent areas and highly electron-dense granules. Arrowheads indicate the two sets of centriolar complexes. Scale bar, 2 μm. B) Late stage of an aberrant spermatid with large electron-dense chromatin globules in the paired nuclei. One nucleus has bead-like breakages of the nuclear membrane (large arrow) and the other has disappearing nuclear membrane (small arrow). Scale bar, 2 μm.

head length through the center on one side of the nuclear disk. The centriolar complex was housed in the articular fossa with the proximal centriole occupying a small indentation at the anterior part, at an angle of 100–120° from the axis of the posteriorly located distal centriole (Fig. 2A). Posterior to the head was a large, collar-like midpiece measuring about 1.8 μm in length and 0.7 μm in width. In the midpiece, a single, fused mitochondrion was observed lying on the cytoplasmic canal surrounding the proximal portion of the tail. The sperm flagellum of the river sculpin was considerably long, measuring about 28.0 μm, and consisted of the usual 9+2 axonemal complex (Fig. 2C).

Aberrant spermatids and spermatid masses. In the testis of the river sculpin undergoing active spermatogenesis, spermatocytes occurred which had doughnut-shaped mitochondria, as well as elongated ones. The doughnut-shape of these mitochondria became more prominent during the later phase of spermatocyte development (Fig. 3A). The nucleus of these spermatocytes was very irregular in shape and contained highly electron-dense chromatin. These cells possessed an intercellular bridge (Fig. 3B) which had multiple disk-like cisternae oriented perpendicularly to the thickened plasma membrane of the bridge (inset in Fig. 3B).

In addition, there were many cysts containing atypical binuclear spermatids (aberrant spermatids) in maturing testis (Fig. 4A, B). In an early phase of their occurrence, the chromatin of paired nuclei in the cells started to condense toward the center of the cell where the respective centriolar complexes were located. The nuclear diameter was about 3.2–4.0 μm. Indentation of the articular fossa, that would house the centrioles, was noticeable. Mitochondria located near the centriolar complexes were spherical in shape. Two flagella, each originating from the respective centriolar complex, had developed. Further nuclear chromatin condensation occurred toward the center of the cell until each nucleus attained a more or less oval-shaped, electron-dense mass. The greater portion of the nucleoplasm in these paired nuclei was electron-lucent. At a later phase, electron-lucent areas and highly electron-dense granules appeared within the dense chromatin mass (Fig. 4A). The electron-dense granules were small and spherical in shape and were probably the core of successively developing 'chromatin globules'. The nuclear membrane facing the electron-lucent nucleoplasm seemed to have started breaking and folding. The nucleus at this stage was about 2.7×3.2 to 3.6×4.1 μm (short axis×long axis) in size.

As spermatogenesis advanced, the electron-dense granules increased in size while the electron-lucent areas shrank. The appearance of a series of bead-like structures as well as the disappearance of the nuclear membrane were observed in the electron-lucent area (Fig. 4B). The developing flagella also had the 9+2 axonemal complex in these aberrant spermatids. At a later phase, although the aberrant spermatids were still enclosed in a cyst, they became irregular in shape. In each nucleus, chromatin globules progressively increased in size, ranging from 0.6 to 0.8 μm. Concomitantly, the size of the nucleus also

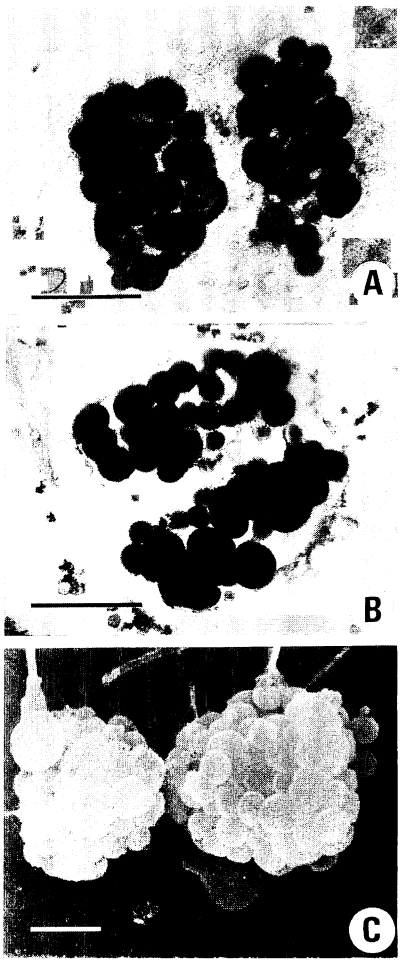

Fig. 5. Electron micrographs of spermatid masses found in the testis of *Cottus hangiongensis*. A) A stage wherein the plasma membrane has disappeared and the organelles are degenerating. Scale bar, 2 μm. B) A stage wherein no organelles could be seen. Scale bar, 2 μm. C) Scanning electron micrograph of a spermatid mass found in the semen collected from the common sperm duct, showing a naked pair of nuclei with large chromatin globules. Note that the globules are smaller than the sperm head. Scale bar, 2 μm.

increased, ranging from 4.2×3.9 to 4.0×4.7 μm, while the nuclear membrane became indiscernible. At this point, these aberrant spermatids (spermatid masses), having two enlarged nuclei with very large globules, were released from their cyst into the seminal lobule lumen. The plasma membrane had broken, and the cytoplasmic organelles began to

degenerate (Fig. 5A, B). These spermatid masses were observed in the common sperm duct as a component of milt, to be subsequently released (Fig. 5C). The globules of the naked pair of nuclei were clearly recognizable and were smaller than the heads of normal spermatozoa.

Discussion

Mature spermatozoa of the river sculpin, *C. hangiongensis*, are quite similar in morphology to those of the marine cottid, *Oligocottus maculosus* (Stanley, 1969), though the sperm head of the latter species is rather slender compared with that of the river sculpin, which is almost disk-shaped. Similarly, the midpiece is large and tapering in *O. maculosus* and two other related species, *O. snyderi* and *O. rubellio* (Fink and Haydon, 1960), whereas it is large and uniform in thickness in the river sculpin. Grier (1981) and Billard (1986) considered the elongated head, and possibly the large slender midpiece, of spermatozoa to be common among fishes with internal fertilization. Most likely, the large midpiece is important for movement of spermatozoa for at least several hours (Grier, 1981; Billard, 1986), as in the case of spermatozoa of *O. snyderi*, which show internal gametic association (Fink and Haydon, 1960). In the river sculpin, although the midpiece is rather large, fertilization is external, probably lasting for a few minutes (Goto, 1988).

In the river sculpin, seminal lobules of the testis during the spawning period were filled with binuclear spermatid masses instead of spermatozoa (Quinitio et al., 1988). The appearance of spermatid masses during spermatogenesis has only been reported in fishes of the family Cottidae. Hann (1927) considered the spermatid masses to have been derived from the fusion of spermatids, which had become aberrant in the course of spermiogenesis. Our ultrastructural observations on the testis of the river sculpin revealed that some spermatocytes became aberrant, having doughnut-shaped mitochondria and nuclei with an irregular contour. These cells also possessed atypical intercellular bridges with multiple disk-like cisternae. Dym and Fawcett (1971) suggested that such multiple disk-like cisternae may develop in the intercellular bridge to prevent the spread of cell death to other cells within the same germinal cyst. In the river sculpin, however, aberrant spermatids connected by atypical intercellular bridges showed synchronous development within the cyst. The atypical intercellular bridge would probably block the passage of information from one cell to another, resulting in the onset of aberration of spermatocytes.

In the later stages of spermiogenesis, binuclear spermatids developed due to incomplete second meiotic divisions, i.e., the nucleus completed the division into two daughter nuclei but cytoplasmic division did not occur. Within the condensed chromatin of the paired nuclei, electron-dense granules occurred, becoming enlarged and forming many globules, which finally packed the nuclei of the aberrant spermatids. The structure of the nuclei of aberrant spermatids was very similar to that of the spermatid masses appearing in the lumen of seminal lobules. Such a mode of chromatin aggregation was never observed in the nucleus of normal spermatids during spermiogenesis. Furthermore, the nuclei of aberrant spermatids became deprived of their envelope before being discharged into the seminal lobule lumen. The change of chromatin characteristics seems to denote some denaturation of the nuclear chromatin leading to death of the nucleus. Several authors have discussed changes of nuclear chromatin in terms of transformation of nuclear basic proteins (Grier, 1981; Billard, 1983). At present, no further explanation can be offered as to the peculiar change in chromatin condensation in the aberrant spermatids of the river sculpin, because of a complete lack of biochemical data concerning nuclear changes during spermiogenesis in cottid fishes.

The findings of the present study strongly support our light microscopic findings that the spermatid masses occurring in the river sculpin originate from aberrant spermatids which became binuclear during spermiogenesis (Quinitio et al., 1988), and that a gradual increase in size of the spermatid masses is apparently due to enlargement of the electron-dense globules of the paired nuclei, but not due to fusion of smaller spermatids or spermatid nuclei, as suggested by Hann (1927) in *C. bairdii*. In the river sculpin, both the aberrant spermatids and spermatid masses were always seen to be binuclear. Nuclei of intermediate sizes between both types of cells were found. Hann (1927) suggested that the masses found in the marine cottid, *Blepsias cirrhosus*, might be formed by the enlargement and degeneration of spermatids without fusion.

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カンキョウカジカ *Cottus hangiongensis* の異型精細胞の出現に関する微細構造的研究

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カンキョウカジカの精子形成と精子変態の過程を，電顕的に調べた．本種の成熟途上の精巣には，ドーナツ状のミトコンドリア，不規則な形状を呈する核，および層板状構造を包含する非典型的な細胞間橋を有する精母細胞を含む包嚢が多数出現した．この精母細胞が，成熟精巣に観察される二核性の精細胞に変わるとみられた．この異型精細胞の対をなした核の内部に多数の高電子密度の小顆粒が出現し，それを中核としてクロマチンの球塊が形成され，その肥大とともに核全体の肥大や核膜の消失などの退行変化が起こり，成熟精巣にみられる特徴的な“精細胞塊”に変形することが確かめられた．

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