Abnormal Spermatogenesis in the Common Liver Fluke (*Fasciola* sp.) from Japan and Korea

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ABSTRACT. Diploid and triploid specimens of Japanese and Korean *Fasciola* sp. showed abnormality in their spermatogenesis. Live germ cells obtained from the testes were observed under a differential interference contrast microscope. In the stages from spermatogonium to spermatid, the cells combined together at the central cytoplasmic bridge during a series of divisions. One spermatogonium becomes a cell group of 8 primary spermatocytes through 3 mitoses. Until the primary spermatocyte stage, cells are divided in a uniform manner. In most of the diploid specimens, the primary spermatocytes are irregularly divided into non-uniform secondary spermatocytes, however, some specimens perform a regular division. In the majority of triploid flukes, the primary spermatocytes are divided in a regular pattern, but some of the specimens perform an irregular division. The non-uniform spermatids do not perform a spermiogenesis. In the diploid specimens, no spermatozoa were found that were produced by spermiogenesis. Whereas in the triploid specimens, some spermatids distributed uniformly on the surface went through a spermiogenesis. We observed some moving spermatozoa in one triploid specimen. The spermatozoa possibly retain their normal reproductive function. — KEY WORDS: chromosome, *Fasciola*, parthenogenesis, spermatogenesis, triploid.

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The valid species of the genus *Fasciola* are *F. hepatica* and *F. gigantica* [9]. Except in some older papers on the chromosomal observation, the chromosome number is 20 (2n=20) in both *F. hepatica* and *F. gigantica*, and both species are believed to be normal zygotes (diploid) [12, 18, 21].

In more recent years, Sakaguchi [13] found diploids [20] and triploids [16] of Japanese *Fasciola* sp. and reported that these specimens lack a pairing of homologous chromosomes in the first meiotic division. The diploids and triploids of *Fasciola* sp. develop parthenogeneticaly, although *F. hepatica* and *F. gigantica* do not. Therefore we believe that *Fasciola* sp. belongs to a group different from both species [27]. Sakaguchi *et al.* [14] found parthenogenetic diploids and triploids in the Korean liver fluke just like in Japanese species. Moreover, Sakaguchi [13] reported an abnormal spermatogenesis in the Japanese *Fasciola* sp., especially through the observation of the nuclei of germ cells, and he concluded that normal spermatozoa are not produced.

In this study, we observed the spermatogenesis process of *Fasciola* sp. from Japan and Korea using living germ cells [3, 6].

MATERIALS AND METHODS

The liver fluke specimens used in this study were collected in the Fukuoka (19 specimens) and the Seoul city slaughterhouses (84 specimens). The specimens for chromosome observation were prepared using the air drying method [22]. One (5.3%) of the 19 specimens from Fukuoka was diploid (2n=2X=20), and remaining 18 (94.7%) were triploids (2n=3X=30). Out of the specimens from Seoul, 65 (77.4%) were diploids and the other 19 (22.6%) were triploids. In a total of 103 specimens, 66 were diploids and 37 triploids (Table 1).

The method used to observe spermatozoa was the following; the specimens were obliquely cut with a razor blade above the center of the body and the contents were pressured out of the testes onto a slide glass. After adding a drop of physiological saline, a cover glass was put on and the resulting preparation was observed using a differential interference contrast microscope.

RESULTS

The results are presented in Figs. 1 and 2. In the testes of *Fasciola* sp. various developmental stages of germ cells were observed. Grouped germ cells were combined together at the central cytoplasmic bridge during spermatogenesis. The *Fasciola* sp. had not so many spermatozoa as did the *Paragonimus westermani* diploids [24]. We could observe just a few spermatozoa during the spermiogenesis and in some cases there were none at all.

Figure 1 shows the spermatogenesis of germ cells found in diploid fluke specimens. "1A" represents a primary spermatogonium, "1B" secondary spermatogonia and "1C"

Table 1. Materials

Locality	Diploid	Triploid	Total
Fukuoka	1 (5.3%)	18 (94.7%)	19
Seoul	65 (77.4%)	19 (22.6%)	84
Total	66 (64.1%)	37 (35.9%)	103

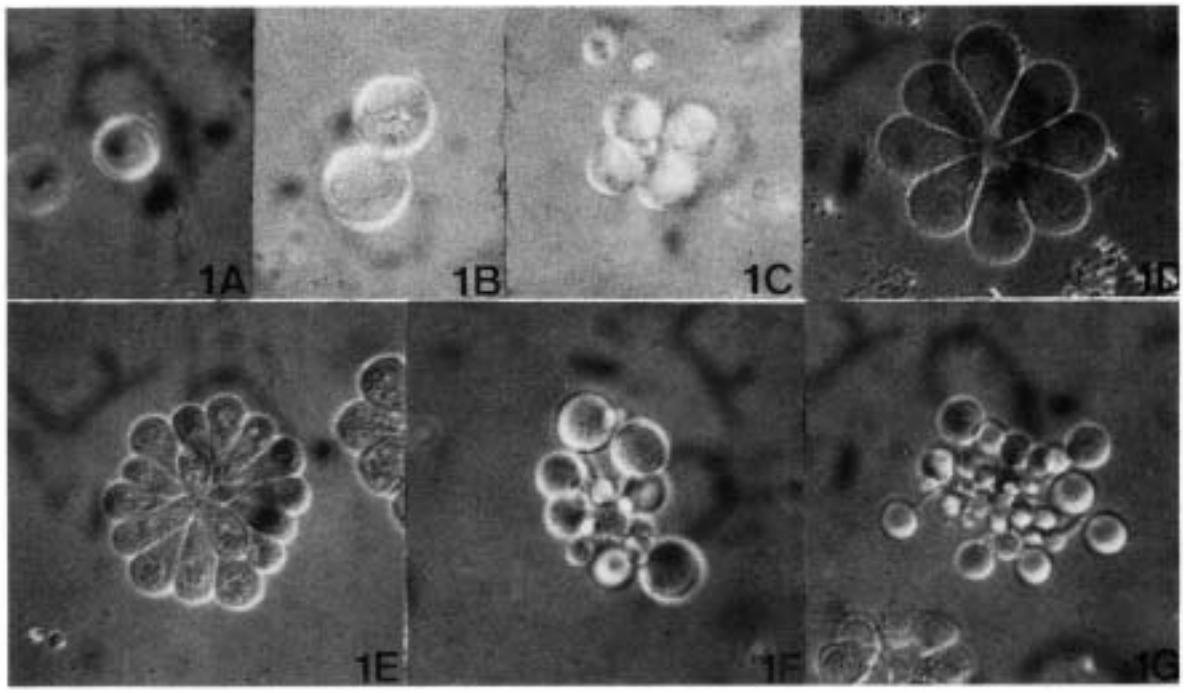

Fig. 1. Various germ cell groups during spermatogenesis in diploid *Fasciola* sp. 1A: A spermatogonium. 1B: Two spermatogonia after a first cell division. 1C: 4 spermatogonia after a second cell division. 1D: Primary spermatocytes in their 8-cell stage. 1E: Secondary spermatocytes in their 16-cell stage, equally distributed on the surface. 1F: Unequally distributed secondary spermatocytes. 1G: Unequally distributed spermatids at 32-cell stage.

tertiary spermatogonia. In "1D", a spermatogonium has divided into 8 primary spermatocytes through three mitoses. The next phase is the first meiosis and results in the formation of a 16-cell group of secondary spermatocytes "1E and 1F". Judging from their appearance, the cells rarely divide uniformly, as seen in "1E". The cells shown in "1G" are a 32-cell group of spermatids and most of them are formed in irregular groups. If spermatogenesis was normally performed as in the *Paragonimus westermani* diploids, the 32-cell group would do a spermiogenesis. But in our observation of *Fasciola* sp. diploids, a spermiogenesis could not be identified, not even in the uniformly divided cells.

Figure 2 shows the spermatogenetic process of *Fasciola* sp. triploids. A spermatogonium in "2A" developed into a secondary spermatogonium "2B" and further into a tertiary spermatogonium "2C". "2D" is a 8-cell group of primary spermatocytes and the process until this stage is similar to that in the diploids. In the case of triploids, as shown in "2E", the secondary spermatocytes (16-cell group) were formed with relative high uniformity in the first meiotic division. In the next stage the secondary spermatocytes were transformed into spermatids (32-cell group) in the second meiotic division (Fig. 2, 2G).

Within the grouped spermatids that are uniformly distributed (Fig. 2, 2I), some cells have a protrusion at the free end, which indicates the start of a spemiogenesis. Flagella appear and grow on both sides of the protrusion. After that, the flagella are reeled up to the protrusion and a "middle snook [6]" is formed. The reduced spermatid body is transformed into a spindle shape (Fig. 2, 2J). The flagella are split at the tip of the middle snook while the original cells lying on the other side of the tip shrink and become quite reduced in size. "2K" shows grouped spermatozoa and within them a number of shrinking cells can be observed. "2L" shows almost fully grown spermatozoa and some of them show movement. However, these spermatozoa are extremely small in number and are observed in one *triploid Fasciola* sp. specimen only.

In comparing triploids and diploids, the number of non-uniformly divided 16-cell secondary spermatocytes is clearly smaller in triploids than in diploids, as shown in "2H". In some of the 16-grouped cells considered as secondary spermatocytes, some signs of spermiogenesis were noticed (Fig. 2, 2F).

Next, we observed the previously prepared specimens for chromosome observation and the results were compatible with these of Table 2. Circular nuclei were found in the diploid specimens, and elongated nuclei suggesting the start of a spermiogenesis could not be found. On the other hand, in the 37 triploids specimens observed, 4 (10.8%) did not show any signs of spermiogenesis, but the remaining 33 (89.2%) showed a change in the shape of nucleus, although the number of spermatozoa was small.

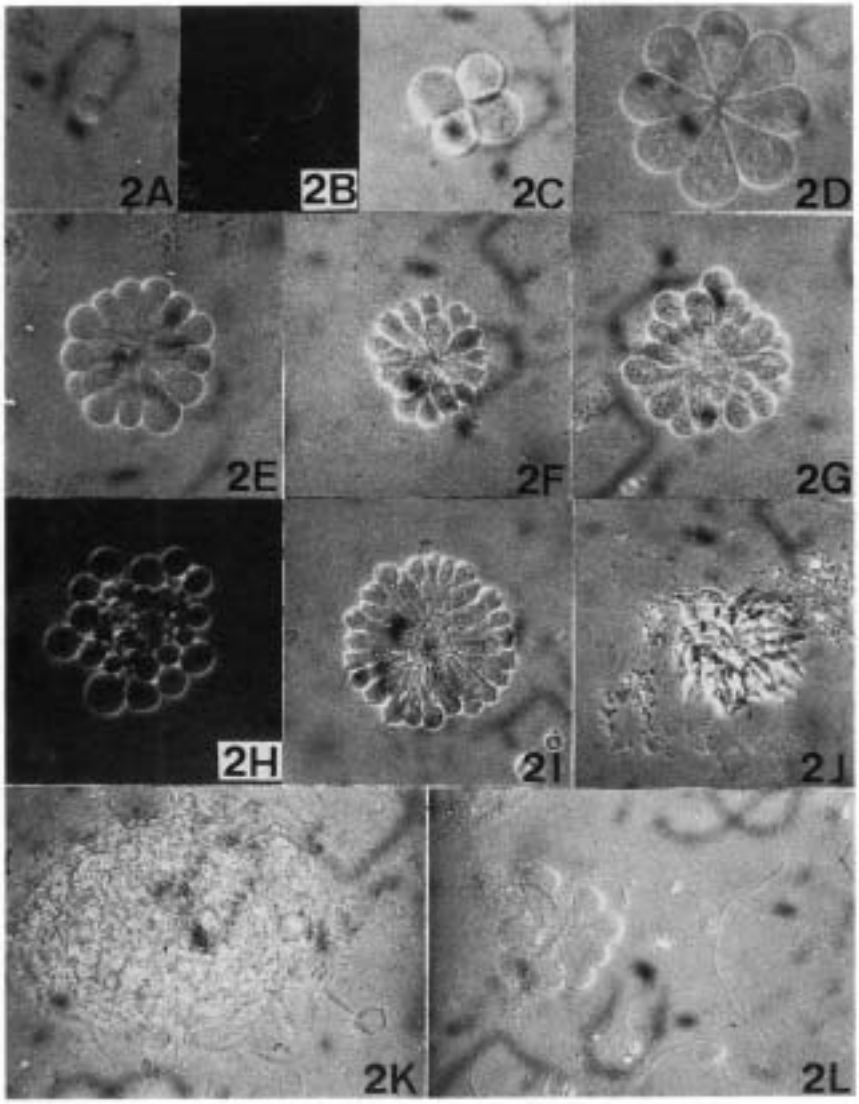

Fig. 2. Various germ cell groups during spermatogenesis and spermiogenesis in triploid *Fasciola* sp. 2A: A spermatogonium. 2B: Two spermatogonia after a first cell division. 2C: Four spermatogonia after a second cell division. 2D: Spermatocytes in their 8-cell stage. 2E: Secondary spermatocytes in their 16-cell stage, equally distributed on the surface. 2F: Secondary spermatocytes in 16-cell groups, initiating a spermiogenesis. 2G: Spermatids in their 32-cell stage, equally distributed on the surface. 2H: Spermatids in their 32-cell stage, unequally distributed. 2I: Spermatids intiating a spermiogenesis. 2J: Grouped spermatids during spermiogenesis. 2K: Spermatozoa. 2L: An intact spermatozoon in movement.

Table 2. Prevalence of sperm nuclei in diploid and triploid specimens for chromosome observation of *Fasciola* sp.

Specimens	Sperm	No sperm	Total
Diploid	0	66	66
Triploid	33 (89.2%)	4 (10.8%)	37
Total	33 (32.0%)	70 (68.0%)	103

DISCUSSION

Numerous studies have been carried out on the spermatogenesis of flukes, for example, Gresson [3] explains the basics of the phenomenon, and there are also many other studies on the spermatogenesis of the genus *Fasciola* [1, 2, 4–7, 29]. These researches were based on observations of the spermatogenesis process and the results agree in most of the points, although differ in some aspects.

In recent years, a triploid of *P. westermani* was discovered [17, 23], which was named *P. pulmonalis* by Miyazaki [10]. Spermatogenesis was compared between the triploid and diploid of *P. westermani* [24, 25]. The spermatogenesis of *P. pulmonalis* is basically similar to that of *Fasciola* sp. in the present study. *P. pulmonalis* from Amakusa, Japan, which was not found to have a spermiogenesis, were similar to the diploids of *Fasciola* sp., whereas *P. pulmonalis* from Tsushima, Japan, had only a small number of spermatozoa in the testes, as the triploid *Fasciola* sp. did.

In this study, we collected diploid and triploid specimens of *Fasciola* sp. from Japan and Korea. It was already recognized that *Fasciola* sp. from Japan and Korea were abnormal in their spermatogenesis [8, 11, 14, 16, 20]. Our present research revealed that the percentages of diploid and triploid specimens are different between Japan and Korea (Table 1). Sakaguchi *et al.* [14] obtained the same results and concluded that no major differences existed between Japanese and Korean *Fasciola* sp. in the size of body and eggs, and also in their cytological features. Therefore both *Fasciola* sp. have been classified in the same category. We agree with their opinion based on the fact that the present research could not make a clear distinction between the specimens collected from Japan and Korea.

In these specimens, one spermatogonium is divided into 8 uniform primary spermatocytes through 3 mitoses (Fig. 1, 1A-1D; Fig. 2, 2A-2D). In most diploid and some of the triploid specimens, the primary spermatocytes produce non-uniform secondary spermatocytes through a first meiosis (Fig. 1, 1F). In the few remaining diploids and many triploid specimens, however, the secondary spermatocytes are uniform in size (Fig. 1, 1E; Fig. 2, 2E). In the next stage, spermatids are produced through a non-uniform cell division in most diploid specimens (Fig. 1, 1G), and through a uniform cell division in most triploids (Fig. 2, 2I). According to Kayano and Cho [8], during the first meiotic division of the spermatogenesis, there is no pairing of chromosomes but a process resembling a somatic division during which the chromosomes are distributed at random.

In *Fasciola* sp., as well as *P. pulmonalis* [24], throughout the process from a spermatogonium to a 32-cell spermatid, the divided cells are combined to each other by the central cytoplasmic bridge and in all grouped cells, the cell division occurs simultaneously [3].

Normally, a spermiogenesis occurs in spermatids, but we could not find spermatids clearly dividing in a non-uniform fashion. In all the diploid specimens, there was no evidence of spermiogenesis. On the other hand, in the triploid specimens, a protrusion appeared on a uniform spermatid at the free end (Fig. 2, 2I) and flagella appeared on both sides of the protrusion. At first, the flagella grew and the protrusion also became elongated. Subsequently, the flagella were reeled toward the protrusion, whereas the cell body reduced its size and became spindle-shape (Fig. 2, 2J). After the middle snook attained its maximum length, the two flagella remaining at the tip of the middle snook became very small. The original cells remaining on the other side of flagella tip changed to an irregular circular pattern (Fig. 2, 2K).

In only one triploid specimen, we could observe moving spermatozoa. It is easy to imagine that they are the same as those observed in the seminal vesicle by Terasaki *et al.* [26]. *Fasciola* sp. with an abnormal spermatogenesis deposits no sperms in the seminal vesicle, or in extremely small quantity. It is unclear if these spermatozoa still maintain their normal reproductive function. The presence of sperm might be one of the reasons why mixoploidy exists in Japanese and in Korean *Fasciola* sp. [11, 13]. Kayano (personal information) established a hypothesis that such mixoploidy was yielded by the fertilization between a diploid egg with two genomes and a triploid sperm with one genome.

No spermatozoa were observed in the diploid specimens, therefore, we can postulate that a spermiogenesis does not occur in diploid specimens, although a more thorough investigation might allow us to find some evidence of spermiogenesis in diploid flukes. In the triploid flukes, on the other hand, a spermiogenesis was observed in most specimens. However, the spermatozoa observed were in small number and showed no movement, except in one case. All these situations seem to cause the abnormality in spermatogenesis of diploid and triploid specimens.

Also, in one of the 16-cell secondary spermatocytes of triploids specimens that showed a uniform cell division, a protrusion was formed at the free end of the cell (Fig. 2, 2F). The same phenomenon happens in *P. pulmonalis* [25], but the meaning is still uuclear.

The abnormal spermatogenesis observed in Japanese and Korean *Fasciola* sp. has also been reported in triploid *Fasciola* sp. specimens from Hawaii [19] and India [15]. Occurence of diploid flukes has not been published except in Japan and Korea. The distribution of diploid and triploid flukes in Asia is interesting. Triploids of *P. pulmonalis* are found only in Asia. In recent years, diploids and triploids of *Fasciola* spp. have been discovered in China [28], but a pairing of homologous chromosomes seems to occur during their meiosis. The presence of tetraploid *Fasciola* has also been suggested. Consequently, the diploids and triploids in China are not considered the same as those in Japan and Korea. More thorough researches on the karyotypes and spermatogenesis of *Fasciola* sp. are necessary.

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