Abstract. In the process of establishment of an inbred Duroc pig strain, males with size asymmetry of the testes were frequently observed. To clarify the possible causes of this asymmetry, we examined the testes and epididymides of 67 males of the F4–F7 generations at 35–100 weeks of age. Testicular weights showed a wide variation (120–610 g). When the weights of the testes were compared bilaterally, 35 of the 67 males showed more than a 10% difference. Histological examination of testes from this asymmetry group revealed a range of seminiferous tubule disruption including disappearance of all germ cells, but not Sertoli cells, in the epithelium. Focal lesions associated with the degenerated tubules were observed. Trends of incident fibrosis or hyalinization of these lesions were seen in aged males of the asymmetry group. Besides this abnormality of spermatogenesis, infiltration of mononuclear inflammatory cells around the tubule was frequently observed. The incidence did not differ between the symmetry and asymmetry groups. Testicular testosterone levels were significantly increased in the asymmetry group, but those of E2 and inhibin did not differ between the two groups. These histopathological features indicate that disruption of spermatogenesis after orchitis/epididymo-orchitis could induce testicular atrophy. Genetic predispositions for this trait may cause prevalent retrograde infections, resulting in orchitis/epididymo-orchitis.

Key words: Orchitis, Pig, Spermatogenesis, Testicular atrophy

Once established at the time of puberty, spermatogenesis is maintained throughout life and depends mainly on the self-renewal ability of spermatogenic stem cells. Spermatogenesis consists of spermatocytogenesis (proliferation and differentiation of spermatogonia into primary spermatocytes), meiosis and spermiogenesis (differentiation of spermatids into sperm). In each process, germ cells are sustained in a favorable environment created by Sertoli cells and other somatic cells under appropriate endocrine and paracrine regulation [1, 2]. Immune privilege in the testis is a current topic of interest in relation to spermatogenesis. Despite the antigenic activity of spermatogenic germ cells and their appearance long after maturation of the immune system, germ cells are protected from autoimmune attack [3, 4]. The milieu surrounding germ cells is prerequisite for sustaining spermatogenesis, and therefore you may say that male reproduction does hang, not stay, in balance.

In livestock and domestic animals, males whose semen quality is obtained periodically and that the boars could become a useful model for allowing a better understanding of reproduction in male animals could be of worth not only for improving efficiency in animal industries but also for understanding the pathogenicity of human reproduction.

In 1998, Gifu Prefectural Livestock Research Institute (Minokamo, Gifu, Japan) began to establish an inbred pig strain from a pair of Duroc pigs. The siblings were mated for several generations basically by brother-sister mating. During this process, males with size asymmetry of the testes were frequently observed. Therefore, we planned to examine the testes and epididymides of the males of all generations histologically to clarify the reasons for this testis asymmetry. The results revealed that incident orchitis/epididymo-orchitis in this strain induced testicular atrophy with impaired spermatogenesis. We consider that there may be genetic predispositions for this trait, and that the boars could become a useful model for allowing a better understanding of reproduction in male animals.©2013 by the Society for Reproduction and Development

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understanding of the nature of immune privilege and inflammation in the testis.

**Materials and Methods**

**Animals and tissue collection**

This survey was undertaken using male pigs that were produced and kept with standard feed for the swine industry at Gifu Prefectural Livestock Research Institute (Minokamo, Gifu, Japan). Siblings were subjected to brother-sister mating, except for a few that were used for crossbreeding between generations to obtain sufficient numbers of siblings. Fluid portions of ejaculated semen were collected from males when they reached reproductive age (approximately 26 weeks) to check the state of spermatogenesis. Boars were sacrificed at slaughterhouses in accordance with the Slaughterhouse Act of Japan. When testes were confirmed to be normal (i.e., not showing cryptorchidism), they were collected along with the epididymis. After trimming off the excess connective tissue, the testes were weighed. A portion of the testes and epididymides was fixed immediately in Bouin’s fixative. For fluoroimmunoassays, another portion of the testis was collected, frozen immediately and then kept at –80°C until use.

**Morphological analysis**

The fixed samples were dehydrated and then embedded in paraffin. Tissue sections 4 μm thick were stained with hematoxylin and eosin or Azan. Immunohistochemistry was undertaken as described previously using anti-cytochrome P450 aromatase mouse IgG (MCA2077S, AbD Serotec, Kidlington, UK) and a Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA, USA). For identification of B cells, sections that had been subjected to a blocking procedure were incubated with biotin-labeled anti-swine IgG (biotinylated goat IgG, Vector Laboratories) for 1 h at 37°C and then reacted with avidin-biotin peroxidase detection kit, as described previously.

**Hormone assays**

Each testis was weighed and then homogenized in 4 volumes of PBS (Dulbecco’s PBS, Nissui, Tokyo, Japan) containing 0.5% CHAPS and protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) at 4°C. After centrifugation at 19,000 × g for 30 min at 4°C, the supernatant was collected and supplied for assays. Concentrations of total inhibin, testosterone and estradiol-17β were measured by fluoroimmunoassay as described previously.

**Statistical analysis**

Hormone assay data are presented as means ± SD. The Student’s t-test was used to compare the assay data between symmetry and asymmetry groups. Differences at P <0.05 were considered to be significant.

**Results**

**General observations**

In this study, 67 adult boars (15 from the 4th generation, 24 from the 5th generation, 12 from the 6th generation, and 16 from the 7th generation) between 35 and 100 weeks of age were analyzed. Semen of the each animal was checked after 26 weeks of age, except for five males. Although one of the 62 males showed a low sperm concentration (0.9 × 10⁸ sperm per ml), the others had normal concentrations (over 1 × 10⁸ sperm per ml), thus confirming that spermatogenesis normally initiated. With regard to sperm viability, three out of the 62 males showed less than 60% viability, and another nine boars showed normal sperm concentrations (2.8–10.0 × 10⁸ sperm per ml), but the sperm were completely immotile.

Testicular weights showed variations. When the weights were compared bilaterally, marked asymmetry was detected (Fig. 1). Testes from 32 of the 67 males showed a difference of less than 10%, but 35 males showed a weight difference of over 10%. Among the 35 males with testis weight asymmetry, the difference was over 20% in 15 of them. The most typical case was testes weighing 380 g and 120 g (a 68% difference between testes) in a 51-week-old male. According to data on cryptorchidism in males of this strain, testis weights have varied between 80 and 180 g, and the mean ± SD has been 116 ± 38 g (n=17). Comparable weights of the asymmetric small testes to those of cryptorchid testes, in which spermatogenesis was completely abolished, seemed to reflect markedly impaired spermatogenesis in the asymmetric testes.

**Histopathology of the testis**

General: Histological examinations revealed variable degrees of disruption in the seminiferous tubules, including a decreased number of germ cell layers in the epithelium, a Sertoli cell-only epithelium and tubule atrophy accompanied by fibrosis. Anatomically, the porcine testicular parenchyma is divided into lobules by fibrous septa, which are cords of connective tissue. In cases of severe spermatogenic disruption, most tubules in a lobule exhibited a lack of germ cells but contained Sertoli cells only in the epithelium. In slight or moderate cases, a few affected tubules were sparsely scattered in the lobules. The degree of impaired spermatogenesis in each testis is shown with symbols in Fig. 1. In most cases, the intertubular area was occupied by Leydig cells, despite presence of affected tubules. Exceptionally, however, infiltration of inflammatory cells was sometimes evident, as described below.

Acute lesions: Severe exfoliation of germ cells, possibly indicating an acute process of germ cell depletion, was detected in pig No. 163 at 36 weeks of age. The weights of the right and left testes were 190 g and 280 g, respectively. In the right testis, sloughed elongated spermatids with a ball shape were observed in the lumina of tubules (Fig. 2A). This progressive disorder was observed throughout some lobules, whereas others contained tubules with various degrees of germ cell loss. Some of these tubules were associated with mononuclear inflammatory cells. Such cells were occasionally present inside the basement membrane, but did not protrude into the epithelium (Fig. 2b). In the left testis, tubules containing only Sertoli cells, and also nearly normal lobules, were observed (Fig. 2B). In the affected lobules, the intertubular region was occupied by inflammatory cells instead of Leydig cells. The ball-shaped sloughed germ cells were observed ipsilaterally in the lumen of the epididymal ducts in the caudal region (Fig. 2C). Poor sperm accumulation in the lumen was evident in the cauda and caput regions of the epididymis on both sides.

Chronic lesions: As seen in the testes of pig No. 609 at 41 weeks of age, a focal lesion with total tubule atrophy in the lobule was observed (Fig. 3A). Tubules in the lesion were small in diameter, irregularly
shaped and surrounded by thickened fibrous septa. Leydig cells had disappeared, and instead, inflammatory cells including neutrophils with a typical nuclear shape occupied the intertubular area. Fibrosis in and around the focal lesion was evidenced by azan staining (Fig. 3B and C; No. 224 at 55 weeks of age). Immunohistochemistry revealed morphologically distinguishable Leydig cells showing a positive reaction with anti-cytochrome P450 aromatase (Fig. 3D), in accordance with previous reports [7, 8]. Cells that were morphologically different from Leydig cells also exhibited a strong positive reaction in the focal lesions. These positive reactions were not absent in the negative control (lacking the primary antibody, data not shown). When the mirror section was incubated with biotin-labeled anti-pig IgG, positive reactions were observed, indicating that these cells were IgG-producing cells, i.e., B lymphocytes (Fig. 3E). We did detect Alcian blue-positive cells, i.e., mast cells, in the testis but not TNF-α-positive cells by immunohistochemistry (data not shown).

Another morphological feature of the focal lesions was hyalinization of the tubules, whose lumina were not easily recognizable and were occasionally filled with an eosinophilic substance (Fig. 3F; No. 537, at 95 weeks of age). Focal damage tended to be evident in relatively small testes (less than 200 g in weight) in the asymmetry group and was sometimes located adjacent to normal lobules.

Cellular infiltration: Besides the above morphopathology, infiltration was observed around the seminiferous tubules. In most cases, one or a few tubules per testicular section were affected. The inflammatory cells were accumulated in part of the tubule periphery (outer part of the peritubular myoid cell layer), and Leydig cells were preserved in the interstitium. Most of the affected tubules seemed to have normal spermatogenesis. With regard to the incidence of cellular infiltration, one out of 64 symmetrical testes (1.6%) and 23 out of 70 asymmetrical testes (32.9%) exhibited cellular infiltration in both faint and severe cases.

**Histopathology of the epididymis**

Epididymides from 67 males (129 samples in total, five samples from four males were not collected) were examined histologically. Normally, sperm were accumulated in the ductal lumen of the caput and cauda epididymis (Fig. 4A and B). The epithelium of the epididymal duct consists of two cell types: principal cells, which are columnar with stereocilia on the free surface, and basal cells,
which are oval or conical cells with sparse cytoplasm (Fig. 4C and D). Besides these two cell types, intraepithelial lymphocytes with round or ovoid dark nuclei and bright cytoplasm were present among the principal cells.

Fewer sperm were accumulated in the lumen of the epididymis when spermatogenesis in the ipsilateral testis was affected. Cellular debris, presumably derived from sloughed germ cells, was also observed in the lumen. Cellular infiltration in the epididymis was observed.

Fig. 2. Acute lesions of epididymo-orchitis. Morphology of the right (A) and left (B) testis and the caput (C) and caudal regions (D) of the right epididymis of male No. 163 (36 weeks old). Most of the seminiferous tubules in A showed progressive degeneration of the epithelium with germ cell desquamation (enlarged in a). Mononuclear cells invaded up to the basal cellular layer of the epithelium (enlarged in b). Leydig cells in the intertubular area seemed unaffected. In B, tubules that contained Sertoli cells and few spermatogonia in the epithelium were observed in the center lobule, possibly at the subacute phase. Inflammatory cells occupied in the intertubular area (enlarged in c). Note that the lobule on the left side seemed normal. Abundant desquamation of germ cells seen in the testis was reflected in the lumen of the caput region of the ipsilateral epididymis (C, inset). No marked changes were observed in the caudal epididymis apart from impaired accumulation of sperm (D). Scale bar = 200 µm.
Fig. 3. Chronic lesions of orchitis. Testicular morphology of males Nos. 609 (41 weeks old, asymmetric smaller testis; panel A), 224 (55 weeks old, asymmetric smaller testis; panels B–E) and 537 (95 weeks old, smaller testis; panel F). A cohort of atrophic tubules showing a decrease in diameter was observed (A, asterisks). The intertubular areas were occupied by inflammatory cells. Neutrophils with unequivocal nuclear morphology were included in the area of infiltration (a, arrows). Atrophic tubules in the focal lesion (indicated by arrows in B–E) were small in diameter, and the lumen structure was not recognizable. Interstitial fibrosis was conspicuous in an azan-stained section (C). Immunohistochemistry with anti-cytochrome P450 aromatase antibody showed disappearance of Leydig cells in the focal lesion (D). Incubation of a mirror section of D with biotinylated anti-pig IgG antibody demonstrated B lymphocytes scattered in the focal lesion (E). Note accumulation of B lymphocytes around the seminiferous tubules (inset of E). Patchy lesion accompanying destruction of the tubules was bordered by a normal lobule (F). Hyalinization of the tubule (b). Scale bar = 200 µm.
Lesions of the epididymis. Morphology of the caput and cauda epididymis of males Nos. 537 (95 weeks old; panels A–D) and 224 (55 weeks old; panels E–G). C–E: Azan staining. Male No. 537 showed a normal morphology in both the caput (A and C) and caudal (B and D) regions. Sufficient accumulation of sperm was seen in the ductal lumen. Arrows in panel C indicate basal cells, which are elemental cells of the epithelium. Typical pathological morphology of the caput epididymis is evident in E–G. Arrows in E and panel a indicate invasion of inflammatory cells into the epithelium. Arrows in F and panel b indicate cuffing of blood vessels with inflammatory cells. Poor accumulation of sperm was evident in the lumen. Scale bars in A, B, F and G indicate 200 µm, and those in C, D and E indicate 40 µm.
in both the symmetry and asymmetry groups (10 out of 62 samples and 13 out of 67 samples, respectively). The inflammatory cells had accumulated outside the periphery of the tunica muscularis or the epithelium (Fig. 4E). In some cases, protrusion of the cells toward the lumen was observed in the epithelium. A cuff of inflammatory cells was observed in blood vessels located near the duct (Fig. 4F and G) in a total of 18 of 129 epididymides.

**Hormone levels**

Testes from eight boars in the symmetry group and 10 in the asymmetry group were subjected to assays of testosterone, estradiol-17β and total inhibin. For reference, four cryptorchid testes and 12 testes from six Large White boars were also employed. The results are shown in Table 1. No significant difference was detected in the testicular concentrations of the inhibin and estradiol-17β, whereas a significant difference was detected in the testosterone concentration between two groups. The individual testosterone concentrations were within a wide range in both groups (45.4–122.2 ng/ml in the symmetry group and 44.2–176.1 ng/ml in the asymmetry group). Relationships between degrees of defect in spermatogenesis and testicular testosterone levels were not detected.

**Discussion**

Histological examinations showed that the marked incidence of testis size asymmetry in this Duroc strain was due to testicular atrophy after sexual maturation. Differences in the weights of the bilateral testes reflected the degree of disruption of spermatogenesis. To our knowledge, this is the first report to describe early regression of spermatogenesis in domestic animals. Sires are required to have sufficient fertility for the reproductive period of their life. Early regression of spermatogenesis negatively influences reproduction and production efficiency. In general, boars are selected as sires after checking of their semen quality. It is assumed that sufficient stress has been placed on whether fertility is maintained, perhaps because of a belief that spermatogenesis remains efficient throughout life. Early regression of spermatogenesis could have been a feature of our Duroc strain, and perhaps has a genetic predisposition. However, it is conceivable that this trait might be a new characteristic that needs to be considered when estimating the reproductive performance of prospective sires.

A tendency for chronic orchitis was observed in the asymmetry group (23 out of 70 testes, 32.9%). Etiologically, infections with viruses, bacteria and protozoa, physicochemical agents, autoimmune mechanisms and other nonclassifiable factors have been indicated as causative of disease [9, 10]. Morphopathological characteristics as well as the frequent incidence of epididymitis in this group are suggestive of infectious orchitis. To date, several swine infections have been shown to cause orchitis or epididymo-orchitis and reduction of semen quality. These include pseudorabies (Aujeszky's virus [11]), porcine rubulavirus ("blue eye" disease virus [12]), porcine reproductive and respiratory syndrome (PPRS) virus [13, 14] and trypanosomiasis due to *Trypanosoma brucei brucei* [15]. However, it is unlikely that these infectious agents would have been responsible for the orchitis in the present study because they are specific pathogens and the boars used in this study had been kept under strictly controlled conditions. Besides these specific pathogens, many sorts of virus have been isolated from the testis and semen of mammals, and some of them have been shown to cause disease [16]. None of the boars examined in this study had suffered symptomatic diseases, but they might have harbored asymptomatic systemic infections with unknown pathogens, and these could have been responsible for orchitis or epididymo-orchitis.

Retrograde infections with common urinary pathogens are another possible explanation for the orchitis/epididymo-orchitis observed in the present study. The prevalence rate of retrograde infection and its influence on male fertility in livestock animals have not been discussed extensively. In humans, inflammations of the testis and other parts of the male reproductive tract are accepted to be an important etiological factor of infertility, and thus nonspecific bacterial orchitis is of some concern [17, 18]. The incidence of epididymitis in boars of the symmetry and asymmetry groups was comparable, possibly reflecting retrograde infection that remained in the epididymis and did not spread up to the testis. Otherwise, the part of the sample examined might have been free of orchitis. Even if this were the case, any influence on the testis in the symmetry group would not have been significant, since the presence of epididymitis was not associated with the weight of the ipsilateral testis (less than 10%).

Noninfectious orchitis is also of some concern when interpreting the present results. The testis is an immune-privileged tissue in which germ cells with antigenic activity are protected against autoimmune reactions [3, 4]. Once this well-organized condition is disturbed by any factors, the activated immune system will begin to attack antigenic germ cells. For example, activation of immune functions after exposition of syngeneic testicular germ cells is suggested to be responsible for orchitis/epididymitis after vasectomy or vas-reversal in humans and nonhuman primates [9, 19, 20]. Allergic orchitis is also incidental to some genetic conditions in mice [21], dogs [22] and minks [23]. Similar conditions of autoimmune orchitis are mimicked in mice by repeated injections of live germ cells without adjuvants. The resulting testicular morphopathology is similar to that which was observed here, although it does have several different features, including deposit of immunoglobulin around the seminiferous tubules [9, 21, 23] and accumulation of inflammatory cells around the rete testis [24–26]. Different influences of activated immune functions in the testis and epididymis might be expressed in a species-specific manner, and thus autoimmune orchitis/epididymo-orchitis must be considered in the present context.

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**Table 1. Concentrations of inhibin, testosterone and estradiol-17β in testes from males in the symmetry and asymmetry groups**

| Group         | Number of testes | Inhibin (ng/ml) | Testosterone (ng/ml) | Estradiol-17β (ng/ml) |
|---------------|------------------|-----------------|----------------------|-----------------------|
| Symmetry      | 16               | 25.4 ± 5.3      | 78.4 ± 20.4          | 15.8 ± 9.8            |
| Asymmetry     | 20               | 25.4 ± 5.6      | 100.6 ± 34.0*        | 16.4 ± 5.5            |
| Cryptorchid   | 4                | 27.4 ± 4.2      | 118.2 ± 54.0         | 19.0 ± 3.1            |
| Large White   | 12               | 21.4 ± 2.9      | 94.6 ± 31.4          | 7.6 ± 3.9             |

Mean ± SD. No significant differences in the three kinds of hormones were detected between the groups. Data for testes from cryptorchid and Large White males are shown for reference. *Significantly different between the symmetry and asymmetry groups (P<0.05).
The severely affected testes exhibited focal lesions of lobules in which all the tubules showed atrophy and fibrosis was observed. As shown in Fig. 3, such lesions were located among normal lobules. This distribution pattern of lesions is suggestive of the features of canalicular infections. We demonstrated the presence of B lymphocytes and mast cells in the lesions (data not shown). The latter cells secrete serine protease tryptase, an enzyme that facilitates fibrinolysis proliferation and collagen production [27, 28], resulting in tubule fibrosis and hyalinization. Abnormal counts and distributions of immune-competent cells have been demonstrated in the testes of infertile men [9, 29, 30]. It is noteworthy that orchitis/epididymo-orchitis tends to lead to scar formation without repair of spermatogenesis. Ludwig et al. [31] demonstrated persistent testicular inflammation in mice that could depend on immune responses activated by initial infectious acute orchitis. Therefore, prevention and early diagnosis of inflammation as well as treatment to eradicate the pathogen are important for maintenance of spermatogenesis.

Asymmetric testes showed higher levels of testosterone and similar levels of estradiol-17β and inhibin compared with the symmetrical testes of infertile men [9, 29, 30]. It is noteworthy that orchitis/epididymo-orchitis tends to lead to infertility, and induces germ cell death by apoptosis. J. Viral 1997; 71: 9170–9179. [Medline] [CrossRef]

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