Original Article

Morphological considerations of the reproductive organs in anestrus gilts affiliated with true hermaphrodites in Thailand

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Abstract

True hermaphrodites are animals with both male and female gonads that have concurrently developed in the same individual. The incidence of true hermaphroditism is moderately higher in pigs than in other domestic animals. The aim of this study was to describe the genital organs of gilts culled due to anestrus with true hermaphrodites by gross and histological analysis. Three anestrus gilts were categorized as unilateral true hermaphrodites, having one ovotestis and one ovary, while three other gilts were bilateral true hermaphrodites with two ovotestes. The anomalous structures of the female genital organs, such as oviductal aplasia and hyperclitoris were found in these gilts. The testicular tissue of all ovotestes was mainly composed of Sertoli cells without any spermatogenic cells and a peculiar proliferation of Leydig cells. The anatomical and histological structures of the ovary, ovarian tissue of ovotestis, oviduct and uterus seemed to be normal in some pigs but the incidence of endometritis, uterine edema and uterine epithelial deterioration occurred in both groups and, in particular, ovarian cysts were usually detected in the bilateral ovotestes. These findings clarify the abnormalities of true hermaphrodite gilts in commercial pig stocks in Thailand and indicate the association between the anomalous development of genital organs and the genetic/hormonal disorders that could be the cause of anestrus.

Keywords: hermaphroditism, morphology, light microscopy, reproductive failure, pig

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Introduction

The important reasons for culling female pigs in commercial swine herds are reproductive disturbance, which consists of vaginal expulsion, anestrus, repeat breeding, no pregnancy and abortion, since these culminate in financial loss to pig production (Dijkhuizen, 1989; Heinonen *et al*., 1998). The intact reproductive function is too intricate to be clinically observed under field surroundings and so the female genital tracts of culled pigs from abattoirs are valuable resources for assessing reproductive aberrations in pig farms (Dalin *et al*., 1997; Koketsu *et al*., 1997; Tummaruk *et al*., 2009). About 49.5% of culled gilts in Thailand present at least one abnormality of their reproductive organs, such as endometritis, ovarian cysts or congenital deviations, in which the latter have been demonstrated in 8.0% of these gilts (Tummaruk *et al*., 2009). After post-mortem examination, congenital abnormalities in culled gilts have been found to be present in different reproductive organs, such as segmental aplasia of the uterus or oviduct, unilateral short oviduct, uterus unicornis, uterus without uterine body and abnormal gonads. The malformation of gonad development in pigs has been reported worldwide and is frequently designated as intersex or intersexuality (Hunter, 1996). Certainly, these intersex pigs usually reveal signs of reproductive failure associated with infertility or sterility before slaughter (Hunter *et al*., 1982). Therefore, the entire reproductive organs of anestrus gilts that appear to show intersexuality traits should be collected and scrutinized to provide valuable information for veterinarians and farmers who work in commercial swine farms.

Intersex animals are considered by the co-occurrence of male and female sex characteristics in the same animal that may be caused by chromosomal abnormalities, hereditary adrenal hyperplasia, fetal exposure to excess sexual hormones and testicular feminization and are known in sheep, goats, cattle and, particularly, in pigs (Hunter, 1996; Lee *et al*., 2013). Intersexuality is characterized in terms of the condition of the sex chromosomes, gonads and external genital organs as true hermaphrodites, male pseudohermaphrodites and female pseudohermaphrodites (Halina *et al*., 1984; Jainudeen and Hafez, 1993). Remarkably, true hermaphrodites are animals that possess gonadal tissue of both genders such as (i) an ovary and a testis, a so-called lateral true hermaphrodite, (ii) an arrangement of a testis or an ovary with an ovotestis, termed as a unilateral true hermaphrodite and (iii) with two ovotestes, named as a bilateral true hermaphrodite (Hunter and Greve, 1996; Pailhoux *et al*., 2001).

Most true hermaphrodites in pigs often have XX chromosomes and the outer sex organs are established in variable degrees relying on the serum testosterone level secreted by the interstitial cells of the testis (Hunter and Greve, 1996). In pseudohermaphrodites, there is a difference between the external genital organs and the true gonad, where male pseudohermaphrodites have two testes but show female external genitalia, whereas female pseudohermaphrodites have two ovaries but reveal male external genitalia (Jainudeen and Hafez, 1993; Pailhoux *et al*., 1994).

The incidence of intersexuality in female pigs is recognized at 0.2-0.5% in European countries (Einarsson and Gustafsson, 1970; Pinton *et al*., 2002), but ranges from 1.0-2.0% of gilts raised in the closed breeding herds of Scotland (Hunter *et al*., 1982). In Thailand, the intersexuality in gilts varies from 0.2-0.5% at slaughter and the majority of them are true hermaphrodites (Tummaruk *et al*., 2009). To our knowledge, although intensive assessments of true hermaphrodites in pigs have been performed elsewhere, there are no published reports predominantly in culled anestrus gilts in Thailand. The purpose of this study was, therefore, to describe the genital organs of gilts culled due to anestrus concurrent with true hermaphrodites using gross anatomical and histological procedures.

Materials and Methods

Animals and gross anatomical investigation: About 120 cross-bred (Landrace × Yorkshire) gilts from four swine commercial farms in the central and eastern region of Thailand were culled due to anestrus at a local abattoirs during 2017-2018. Six of them displayed the indications of true hermaphrodites and their reproductive organs were obtained, kept in an ice container and delivered to the laboratory within 6-8 h before investigation. Entire female genital organs from these true hermaphrodite gilts were observed by a pathologist and recorded for normal or abnormal characteristics in the same manner as when performing a necropsy report.

Leukocyte culture and karyotype analysis: Blood samples were collected from the external jugular vein of the gilts prior to slaughter and kept in a heparin vacuum tube (Vacutainer®, Becton Dickinson, NJ, USA). Each sample was subsequently centrifuged at 1,200 rpm (258 × g) for 10 mins and then 0.5-1.0 mL of the buffy coat was collected and cultured in RPMI 1640 growth medium (Seromed, Berlin, Germany) supplemented with 15% of fetal calf serum, 2 mM L-glutamine, 0.8 µg/mL of pokeweed, and 1% antibiotic and antimycotic (ABAM; Invitrogen, San Diego, CA) for 3 days at 37 °C, 5% CO_2 and 95% humidity. Forty-five minutes before harvesting, the cells were treated with 0.025 µg/mL of Colcemid (Seromed, Berlin, Germany) that was added and incubated as above for 50 mins to arrest cell division at the metaphase stage. Next, hypotonic 60 mM KCL solution was added and the cells were maintained at 37 °C for 20 mins. The swollen cells were then immersed in cold 3:1 (v/v) methanol: acetic acid fixative at 4 °C for 30 mins before harvesting by centrifugation at 1,200 rpm (258 × g) for 10 mins. The fixed cells were placed on wet glass slides and allowed to air-dry. They were then stained with 5.0% (v/v) Giemsa staining for 10 mins, washed in distilled water and between 100-150 cells were examined and counted under light microscopy (400×). Giemsa bandings from selected lymphocytes (about 15-30 cells) were examined on the metaphase spreads.

Tissue preparation for light microscopy: The gonads from true hermaphrodite gilts were removed from the genital tracts and fixed in 10% neutral buffered formalin for at least 24 h. The samples were then processed using an automatic tissue processor (Tissue-Tek, Sakura, Tokyo, Japan), embedded in a paraffin block and cut into 5-µm thick sections using a microtome (Shandon, Anglia Scientific Instrument, Cambridge, UK). The tissues were deparaffinized in xylene, passed through different concentrations of ethanol and stained using hematoxylin and eosin (H&E). They were then observed under light microscopy (BX50 Olympus, Tokyo, Japan) and photographed with a digital camera (Micropublisher 5.0, Qimage, Surrey, Canada). The tissue micrographs were analyzed using the Image Pro®Plus 6.0 software (Media Cybernatics, MD, USA).

Results

Karyotype and gross anatomical investigations: All six animals in the present research represented genetic females with XX chromosomes and 36 autosomes (38, XX), as shown in Figure 1. The six gilts in this study were culled due to their reproductive failure (anestrus) and the appearance of being a true hermaphrodite. At autopsy, three samples revealed one normal ovary on the left side and one ovotestis on the right side and so were unilateral true hermaphrodites (Fig. 2a), whereas the other three gilts demonstrated two ovotestes and so were bilateral true hermaphrodites (Fig. 2b). A normal bicornuate uterus was present in all animals and at least one pathological lesion, such as pyometra, uterine edema or endometritis, was found in these gilts (Fig. 2b, c). The ovotestes of the true hermaphrodite gilts were transversely cut to illustrate the difference between the testicular and ovarian tissues of these organs (Fig. 3a, b). Interestingly, ovarian cysts were regularly found in ovotestes of the three bilateral true hermaphrodite gilts (Fig. 3c, d), while they were not found in the right ovotestis of the unilateral true hermaphrodite animals.

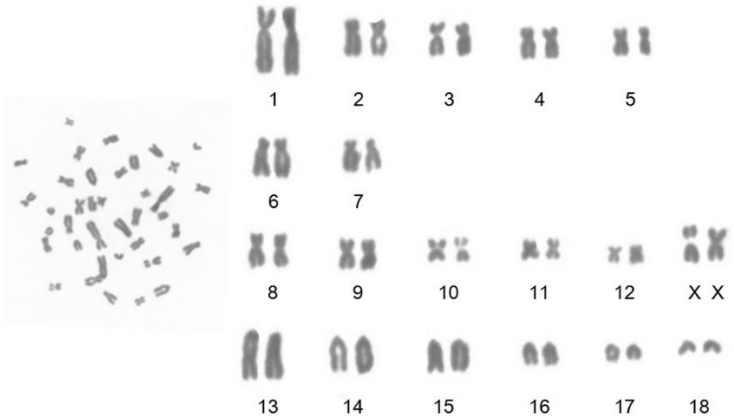

Figure 1 Representative karyotype (left) and karyological idiogram (right) from leukocyte of true hermaphrodite gilts demonstrating 18 pairs of autosomes and one pair of sex chromosomes.

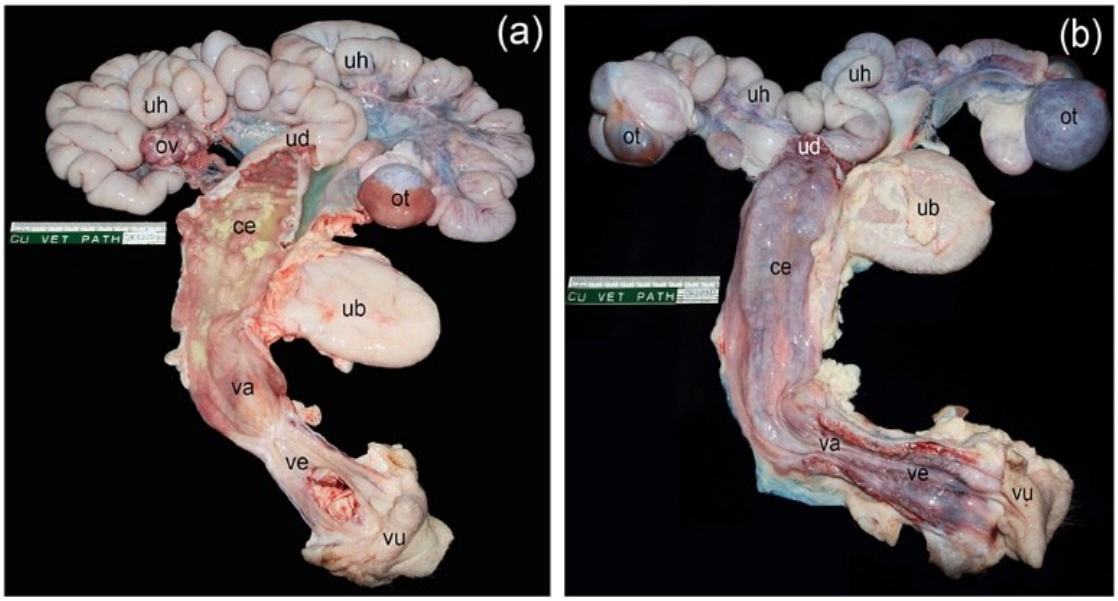

Figure 2 Gross morphology of the reproductive tract and gonads of true hermaphrodite gilts presenting left normal ovary and right ovotestis in unilateral true hermaphrodite gilts and pyometra in cervix (a) and both ovotestes in bilateral true hermaphrodite gilts with the appearance of uterine edema and hemorrhage in the vagina and vestibule (b). ov = ovary; ot = ovotestis; uh = uterine horn; ud = uterine body; ce = cervix; va = vagina; ve = vestibule; vu = vulva; ub = urinary bladder.

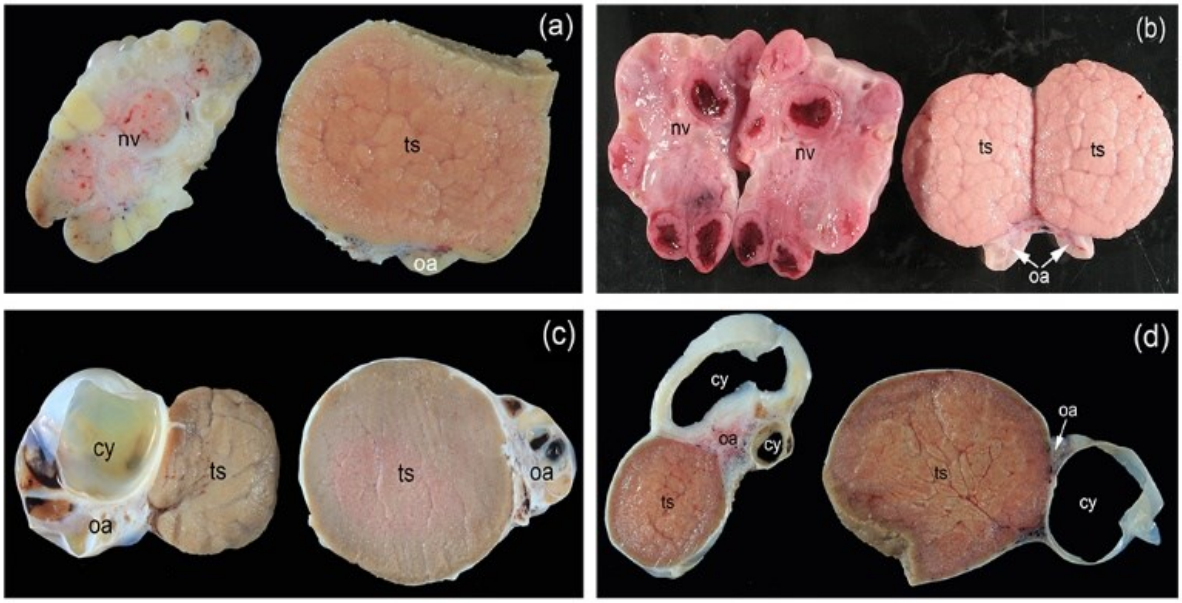

Figure 3 Transverse sections of left ovary showing cyclic changes as a normal ovary and right ovotestis of unilateral true hermaphrodite gilts (a, b) containing predominant testicular tissue and minor ovarian tissue (white arrows) at the peripheral area. Ovotestes of bilateral true hermaphrodite gilts demonstrated the presence of an ovarian cyst on one side of the ovotestis (c) or both ovotestes (d). nv = normal ovary; ts = testicular tissue; oa =ovarian tissue; cy = ovarian cyst.

In two of the three unilateral true hermaphrodite gilts, aplasia of the right oviduct was found (Fig. 4a), whereas no abnormal structures were found in this area but an epididymis-like structure existed and adhered to the right ovotestis of two of the three bilateral true hermaphrodite gilts (Fig. 4b). In addition, the caudal portion of this organ close to the uterine horn in these animals was observed and its features resembled the ductus deferens but this was not clear during autopsy (Fig. 4b). The vulva was thick, narrow and its ventral commissure pointed dorsally like a fish-hook (Fig. 2), while an obvious enlarged clitoris (hyperclitoris) was found in all six animals (Fig. 4c, d). The hyperclitoridis extended from the ventral commissure of the vulva and varied in both size and length in both types of the true hermaphrodite gilts.

Histological analysis of gonads and genital tracts: The main compartment of the ovotestis collected from the true hermaphrodite gilts was testicular tissue, which roughly imitated the normal testicular tissue from male testis, whereas the smaller portion of tissue was the ovarian tissue that was usually situated at the peripheral area of this abnormal organ and was separated by typical tunica albuginea (Fig. 5). The follicular states during follicular growth (Fig. 5a) or the changes into corpus lutea (Fig. 5b) can be observed in the ovarian tissue of these ovotestes. Numerous seminiferous tubules were clearly found in the testicular tissue and, remarkably, the epithelia of the seminiferous tubules depicted somatic cells without any spermatogenic cells at a higher magnification (Fig. 5c, d). Histologically, these cells were more characteristic of Sertoli cells, as detected in normal testicular tissue, and the interstitial tissues were crowded with swollen, strongly eosin-staining cells in the cytoplasm that resembled interstitial or Leydig cells (Fig. 5d).

In the unilateral true hermaphrodite gilts, the normal follicular development of primordial, primary, secondary, tertiary and Graafian follicles was present in the ovarian tissues of all ovotestes (Fig. 6a) and was comparable to that in the normal left ovaries (Fig. 6b). In the bilateral true hermaphrodite animals, normal ovarian dynamics within the ovarian tissue of the right ovotestis was found in one gilt, whereas the major atypical feature of ovarian cysts was frequently detected in the ovarian tissues of all the ovotestes in this group (Fig. 6c-d). Noticeably, the characteristics of the Graafian follicle in the normal ovary of the unilateral gilts appeared healthy and consisted of a complete granulosa layer, theca interna and theca externa (Fig. 6e), whereas alterations in the theca layers were observed in the ovarian tissue of the unilateral ovotestis (Fig. 6f).

The ductus epididymis vicinity to the ovotestis in the true hermaphrodite gilts seemed to be normal as it was lined by an intact pseudostratified columnar epithelium with stereocilia and without any spermatozoa in the lumen (Fig. 7a, b). However, deterioration of these epididymal epithelia was seen in some animals (Fig. 7c, d). Moreover, the ductus deferens was also observed near to the uterine horn in the ovotestis of both types of true hermaphrodite gilts (Fig. 8a), which was confirmed by the appearance of a pseudostratified columnar epithelium with short stereocilia, a thin layer of loose connective tissue and a thick tunica muscularis (Fig. 8b). The section of the uterine horn from these gilts established the endometrium (uterine epithelium and propria-submucosa), myometrium and perimetrium (Fig. 8c). The uterine epithelium varied from a simple columnar type to a pseudostratified columnar type according to the cyclic changes, whereas the propria-submucosa contained superficial and deep endometrial glands. Conspicuously, the purulent exudate within the uterine lumen that might have occurred by

'endometritis' (Fig. 8c) and the abnormal features, such as uterine epithelial degeneration, high leukocyte infiltration and blood congestion (Fig. 8d), were noticed in relation to the pathological lesions found in the uterine horns of these gilts. Interestingly, the portions of the oviduct (isthmus and ampulla) in the left side of the unilateral group were sectioned (Fig. 8e) and the degenerated features, such as epithelial corrosion, luminal atypical debris and blood congestion, were seen in this unilateral gilt (Fig. 8f).

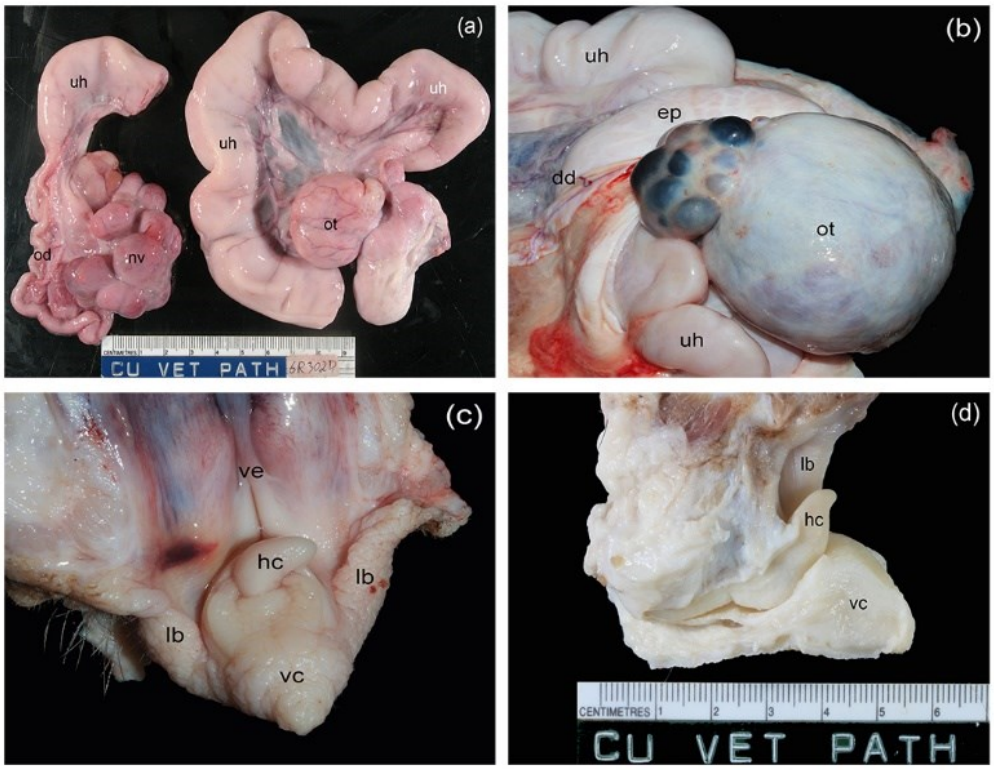

Figure 4 Gonads and reproductive tract abnormalities in true hermaphrodite gilts revealing aplasia in the oviduct on the right side with ovotestis compared to left normal oviduct and ovary (a) and the location of epididymis- and ductus deferens-like structures close to right ovotestis and uterine horn (b), while the hyperclitoris protrudes from the vulva in dorsal (c) and medial (d) views. nv = normal ovary; ot = ovotestis; od = oviduct; uh = uterine horn; ep = epididymis-like structure; dd = ductus deferens-like structure; hc = hyperclitoridis; ve = vestibule; lb = labium; vc = ventral commissure of vulva.

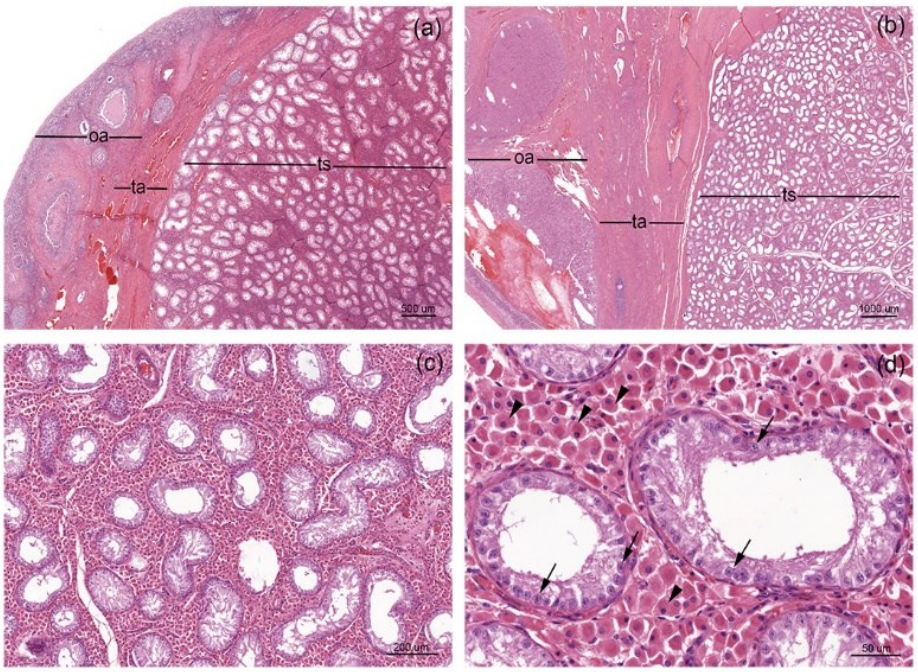

Figure 5 Ovotestes in true hermaphrodite gilts (a, b) showing ovarian tissue and testicular tissue separated by the tunica albuginea. Numerous seminiferous tubules with varied diameters of lumen appearing within testicular tissue (c), and the epithelium of seminiferous tubule composed of Sertoli cells (arrows) and crowded Leydig cells exist in the interstitial tissues of true hermaphrodite gilts (d). oa = ovarian tissue; ts = testicular tissue; ta = tunica albuginea.

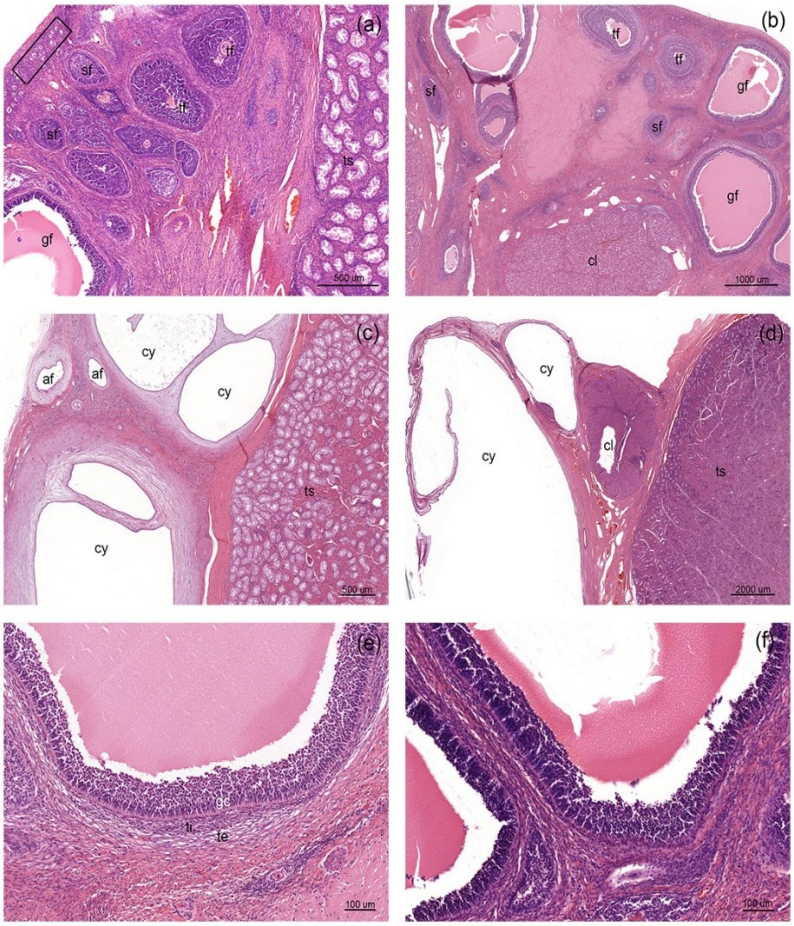

Figure 6 Ovarian tissues in unilateral true hermaphrodite ovotestis showing primordial follicles, primary follicles (in the rectangle) and all follicular states (a) and likewise in the left normal ovary (b). Ovarian cysts and atretic follicles found in ovarian tissues of bilateral true hermaphrodite ovotestes (c, d). Normal structure of Graafian follicle existing in the ovary (e) and in the ovarian tissue of ovotestis (f). sf = secondary follicle; tf = tertiary follicle; gf = Graafian follicle; cl = corpus luteum; cs = ovarian cyst; af = atretic follicle; ts = testicular tissue; gc = granulosa cells; ti = theca interna; te = theca externa.

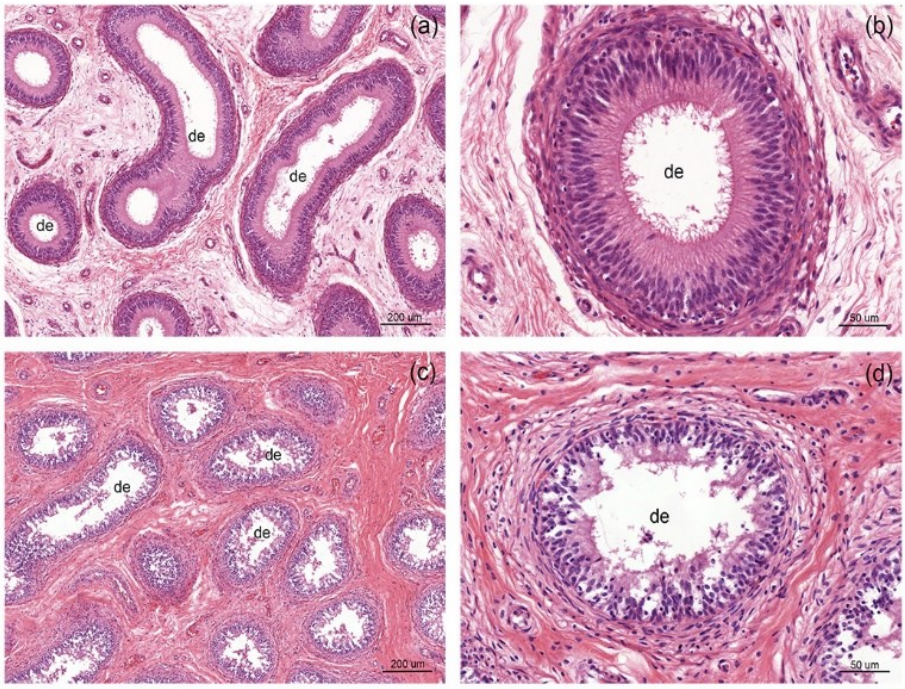

Figure 7 Histological sections of the ductus epididymis of most true hermaphrodite gilts at low (a) and high (b) magnifications showing the normal pseudostratified columnar epithelium with stereocilia without any spermatozoa in the lumen whereas the sections of epididymis from two gilts at low (c) and high (d) magnifications show the epithelial corrosion of these tubes. de = ductus epididymis.

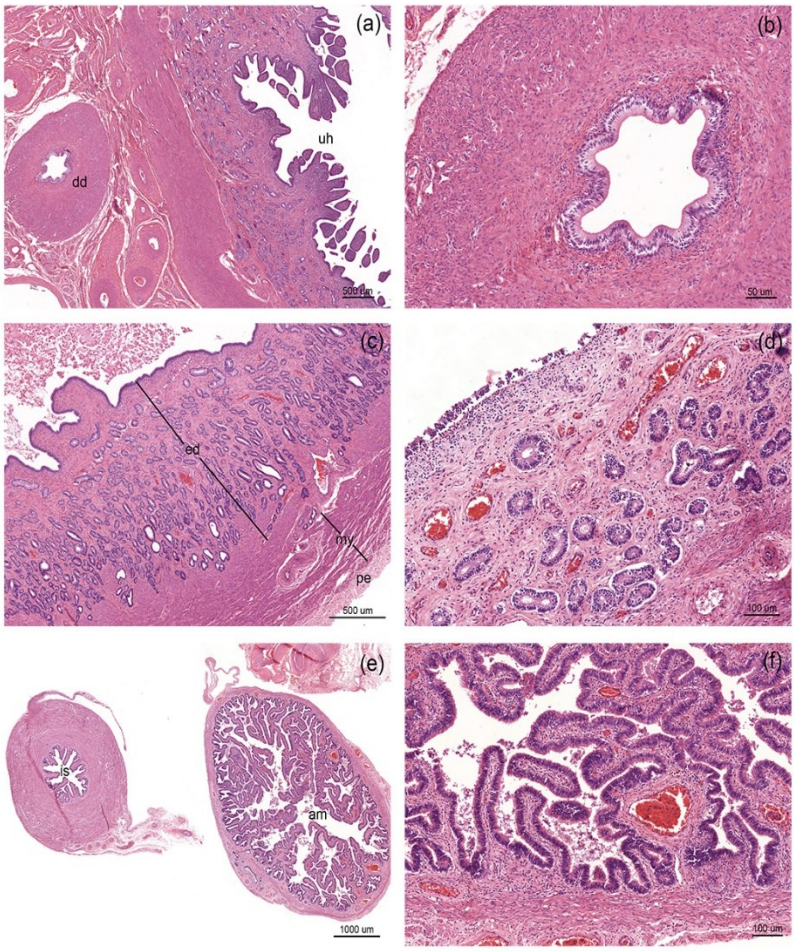

Figure 8 Histological sections of the ductus deferens of true hermaphrodite gilts (a) showing the pseudostratified columnar epithelium and thick tunica muscularis (b). The three layers of uterine horn with a purulent exudate within its lumen (c), and uterine epithelial degeneration, high leukocyte infiltration and blood congestion were found in most true hermaphrodite gilts (d). The isthmus and ampulla in the oviduct of the unilateral group (e) were cut and deterioration aspects were observed in the ampulla (f). dd = ductus deferens; ed = endometrium; my = myometrium; pe = perimetrium; is = isthmus; am = ampulla.

Discussion

The purpose of this study was to use gross anatomical and histological analyses to evaluate the morphology of the female reproductive organs collected from gilts culled due to anestrus and the presence of anomalous lesions of true hermaphrodites in Thailand. We confirmed that the various abnormalities of the genital organs found in true hermaphrodite gilts certainly interrupted the normal reproductive function and might be one of the major factors initiating the anestrus in replacement gilts in commercial Thai pig farms.

Six intersex gilts were examined for their type of true hermaphrodites, in which only two subgroups were found: unilateral and bilateral true hermaphrodites, using the previously reported categories (Hunter, 1996), whereas the lateral true hermaphrodite subgroup was not found in these culled gilts. Both groups of true hermaphrodite gilts demonstrated a female karyotype (38, XX) without any chromosomal variations, corresponding with a previous study (Hunter and Greve, 1996). By anatomical analysis, the manifest bicornuate uterus was observed in all gilts, as reported previously (Hunter *et al*., 1982; Hunter, 1996; Bansal *et al*., 2005; Lee *et al*., 2013), suggesting that the uterine development deriving from the main part of the paramesonephric ducts or "Müllerian ducts" in true hermaphrodites was not disturbed by the existence of testicular tissue on the ovotestes. In contrast, aplasia of the oviduct present in these gilts, indicating the atypical modification of the distal part of the paramesonephric duct, was influenced by factors produced by the ovotestis during embryogenesis. As shown in the present results, the component of ovotestes in the true hermaphrodite gilts was the predominant testicular tissue with relatively scarce ovarian tissue and, interestingly, the wall of the seminiferous tubule within the testicular portion was lined by "Sertoli cells" without any spermatogenic cells, which is in accord with previous reports (Hunter *et al*., 1982; Halina *et al*., 1984; Bansal *et al*., 2005; Lee *et al*., 2013).

Considering the testicular tissue in the ovotestes, the primary factor associated with development of the embryonic gonad into testis in male animals is determined by the SRY gene on the Y chromosome (Inomata *et al*., 1993; Tata *et al*., 2018). In the presence of a functionally expressed SRY gene, establishment of the testis involves the transformation of primitive sex cords enclosing germ cells and Sertoli cells after the differentiation of Leydig or interstitial cells (Jost *et al*.,

1973). In the present results, we found the existence of testicular tissue within the ovotestes in all true hermaphrodite gilts, which all exhibited (38, XX) female chromosomes. However, the absence of a SRY gene on the Y chromosome has been detected in the majority of true hermaphrodite pigs (Lee *et al*., 2013; Pailhoux *et al*., 1997), indicating that the development of male reproductive organs is able to take place in animals without a SRY gene.

The question then still rises as to how the primitive gonad of the (38, XX) true hermaphrodite pigs can develop into unilateral or bilateral ovotestes. Besides the SRY gene, the autosomal gene *SOX9* (SRY-related high mobility group-box gene 9), which is found on chromosome 12 in pigs (Lahbib-Mansais *et al*., 1997), seems to be associated with gonadal differentiation and plays an important role in the differentiation of Sertoli cells from somatic precursor cells in the gonad, which is the preliminary step in testis formation (Sekido and Lovell-Badge, 2009). In pig embryos, the expression of porcine SOX9 is initiated at 21 d post-conception in both genders and its expression increases in the testis and decreases in the ovary from 28 d post-conception, the critical time for gonadal modification in pigs (Parma *et al*., 1999). Moreover, mutations of *SOX9* account for human XY sex reversal (Foster *et al*., 1994) and the inaccurate expression of the *SOX9* transgene in XX mice is able to induce female-to-male sex reversal (Vidal *et al*., 2001). Recently, the expression level of *SOX9* was reported to be increased in the testicular tissues of true hermaphrodites, whereas the level in ovarian tissues of these pigs remained at a normal female level (Rousseau *et al*., 2013). Therefore, from the above assessments, ovotestis formation could be associated with the influence of at least one recessive gene on the porcine autosomal chromosome that is stimulated in unsuitable conditions. In addition, the present results found the disappearance of spermatogenic cells in the seminiferous tubules within the ovotestes of all true hermaphrodite gilts. In the case that the primordial germ cells are able to migrate into the undifferentiated gonad, the explanation of this phenomenon might relate to the increased capability of germ cells to undergo apoptosis because of an intrinsic defect, the absence of a suitable ovarian microenvironment and the existence of a meiosis-preventing substance or AMH (Behringer *et al*., 1990; Hunter, 1996).

Other aspects that were prominently found in the true hermaphrodite pigs were an incomplete epididymis and ductus deferens near to the ovotestis as well as the hypertrophic clitoris. It is known that the Wölffian duct generally forms the epididymis and ductus deferens in the male, whereas the genital tubercle transforms into a penis in males or a clitoris in females. Certainly, the Leydig cells of the fetal testis secrete testosterone to stimulate the development of the Wölffian ducts and the male external genital organs (Inomata *et al*., 1989; Josso *et al*., 1977). In this study, the proliferation of Leydig cells was noticed in the true hermaphrodite gilts, while it was previously reported in canine ovotestis that P450c17, the enzyme mediating androgen-synthesis activities, was detected in Leydig cells (Groppetti *et al*., 2012). Thus, it can be assumed that the androgen levels during fetal growth and at puberty were inadequate to provide the complete establishment of entire structures from the Wölffian ducts and to complete male external organs (Pailhoux *et al*., 2001), corresponding to the present results, where we only found epididymal- and ductus deferens-like structures as well as the hyperclitoris.

Apart from the congenital abnormalities of the gonads and genital tracts, the main pathological lesions found in all true hermaphrodite gilts in the present study were the ovarian cysts. Undoubtedly, anestrus is the major reason for reproductive failure in gilts and sows and causes subfertility or infertility and noteworthy economic loss to porcine herds around the world (Heinonen *et al*., 1998). The pathogenesis of the ovarian cysts that occurs in both humans and animals is multifactorial. For example, it can be caused by stress, hormonal disorders disturbing the hypothalamo-hypophyseal axis (Scholten and Liptrap, 1978), steroidogenic enzymes (Kozlowska *et al*., 2009) and also apoptosis and cell proliferation (Sun *et al*., 2012). Although, there are no reports attempting to investigate the relationship or etiology of ovarian cysts in hermaphrodite animals, the high incidence of ovarian cysts found in this study could be associated with the aberrant gonads of the true hermaphrodite gilts. Previous studies have shown that polycystic ovarian syndrome is due to the alteration of androgenic dynamics (Morgante *et al*., 2015), such as the increased presence of 5 α-reductase, the enzyme that converts testosterone to dihydrotestosterone (DHT) (Vassiliadi *et al*., 2009) or the androgen produced by a Sertoli-Leydig cell tumour (Kamani *et al*., 2019). The AMH serum levels are higher in women with polycystic ovarian syndrome than in normal fertility women, while AMH in gestational hyperandrogenism triggers a masculinization of both the fetal testosterone and a luteinizing hormone surge in female offspring (Tata *et al*., 2018).

In conclusion, this study is the first to investigate the morphological features of the reproductive organs of anestrus gilts with lesions of true hermaphrodites in Thailand. These findings lead us to a better understanding of true hermaphrodite gilts, at least in commercial pig stocks in Thailand, and indicate the correlation between the anomalous development of genital organs and the genetic/hormonal disorders that could be the cause of anestrus, one of the major problems of reproductive failure in replacement gilts. Definitely, the immunolocalization of sex hormonal receptors and AMH in the ovotestes and the sex hormonal levels in blood plasma of these gilts require to be further investigated.

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