Original Article

Anti-Müllerian hormone immunolocalization in growing follicles of replacement gilts culled due to anestrus

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Abstract

In female pigs, the anti-Müllerian hormone (AMH) produced by granulosa cells of developing follicles plays an important role in ovarian reserves of follicles and serum AMH levels are the biological marker of gilt reproductive performance. In the present study, we determined the AMH expression during follicular growth in replacement gilts culled due to anestrus by immunohistochemical technique. A total of 20 reproductive organs from crossbred Landrace × Yorkshire gilts were collected from swine commercial farms in Thailand and categorized by the reasons for culling, i.e., the anestrus group (n=12) and other causes not related to reproduction as the control group (n=8). As expected, the AMH immunolocalization in control gilts was markedly observed in most granulosa cells of healthy preantral, early antral, large antral and preovulatory follicles. The AMH immunostaining in the granulosa cells of most presumably healthy follicles in anestrus gilts depicted weak immunoreaction and the intensity score was significantly lower (P<0.05) than the control gilts. Certainly, the granulosa cells within the atretic follicles in both the control and anestrus groups were faint from AMH immunoreaction. The findings indicate that the low intensity of AMH immunoexpression found in the granulosa cells might be associated with a decreasing of serum AMH level in anestrus gilts. This phenomenon confirms that the insufficient physiological function of AMH during follicular growth is relevant to the cause of anestrus in replacement gilts.

Keywords: Anti-Müllerian hormone, immunohistochemistry, ovary, pig, anestrus

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Introduction

Reproductive disorders in commercial swine farms, for example anestrus, repeat breeding, no pregnancy and abortion are critical problems in removing replacement gilts raised in many countries (Koketsu *et al*., 1997). Importantly, the main reason for culling gilts in Thailand is anestrus and more than 50% of all culled gilts demonstrate normal genital organs at post-mortem examination (Tummaruk *et al*., 2009). Subsequently, microscopic investigations of the reproductive organs collected from culled pigs at abattoirs are necessary as diagnostic tools to identify the abnormalities in these pigs. As demonstrated in earlier studies, the uterus and oviduct of pigs culled due to reproductive disturbances were analyzed and corrosion of luminal epithelium, an increasing of leukocytes and an imbalance in the immunolocalization of sex hormonal receptors was established (Dalin *et al*., 2004; Karveliene *et al*., 2007; Tienthai and Tummaruk, 2015; Tienthai *et al*., 2010). Since the ovaries are the most vital reproductive organ involved in the regulation of follicular growth, oocyte production and steroid hormonal secretion during estrous cycle, these organs must be investigated for disorders in the culled pigs. The immunohistochemical technique was operated to appraise the ovarian disorder, such as cystic ovaries, to describe the variations in growth factors and the imbalance of apoptosis in the developing follicles (Sant'Ana *et al*., 2015; Sun *et al*., 2012; Wang *et al*., 2015). Up to date, the immunoexpression of AMH, however, has not been accomplished yet in the ovaries of replacement gilts culled for reproductive failure.

In mammals, AMH is a member of the transforming growth factor-ß (TGF-ß) and it is originally recognized as a product of Sertoli cells in male fetal testes (Cate *et al*., 1986). The noticeable function of AMH is to induce the regression of Müllerian ducts during testicular development (Munsterberg and Lovell-Badge, 1991). In females, AMH is not found during sex development but it exists in newborn ovaries and it is definitely synthesized by the granulosa cells of the follicles that are indicative of the additional physiological functions of this substance in adult females (Bezard *et al*., 1987; Josso, 1986). It was believed that AMH has two main regulatory steps during follicular growth, i.e. the initial follicle recruitment and the selection of the dominant follicle (Durlinger *et al*., 2002), therefore the serum AMH levels were utilized as a marker for ovarian aging and ovarian responsiveness in humans (Visser *et al*., 2006). This extraordinary concept has been widely applied in ruminants revealing the number of antral follicles definitely correlates with serum AMH concentrations (Baldrighi *et al*., 2014; Batista *et al*., 2014; Maculan *et al*., 2018). In women, the low serum AMH levels are related to a decrease of AMH immunoexpression in the follicles occurring in premature ovarian failure patients (Méduri *et al*., 2007). Recently, circulating AMH concentrations were investigated in pigs in which the lowest AMH levels existed in anestrus gilts compared with gilts demonstrating normal estrus from the commercial swine farms in Thailand (Am-In *et al*., 2020). The immunoreaction of AMH was investigated in healthy porcine ovaries (Kelsey *et al*., 2011; Almeida *et al*., 2018) presenting the normal pattern of AMH in ovarian follicles but AMH immunoreaction was not performed in culled pigs. Therefore, the purpose of this study was to investigate the AMH immunolocalization of growing follicles in anestrus gilts from commercial swine farms in Thailand.

Materials and Methods

Herd management and animal tissue collection: All swine herds in this study were breeding herds with 900-1,200 sows per herd. The gilts entered the gilt pool at a bodyweight of 80-100 kg and were housed in an open housing system with equipment to reduce the impact of high temperatures. In all herds, the gilts were kept in groups of 6-15 gilts/pen with a space allowance of 1.5-2.0 m^2/gilt. Gilts were group-fed a diet formulated to provide 3,200 kcal/kg and 15% crude protein allowing up to 2.5 kg/pig/day, with water supplied ad libitum from water nipples. From 150 to 200 days of age, the gilts had direct contact with mature boars for at least 15-20 mins/day to stimulate the onset of estrus. Generally, the herd management recommended breeding the replacement gilts from 32 weeks of age onwards at the second or later estrus and at a body weight of at least 130 kg. Twenty genital organs of randomly chosen culled crossbred Landrace × Yorkshire replacement gilts from three commercial swine herds in Eastern Thailand were collected from the slaughterhouse. The reproductive organs were retained in ice foam packaging and promptly delivered to the laboratory within 12 h of culling. All the gilts were classified into two groups composed of the anestrus group (n=12) and other causes not involved in reproductive problems as the control group (n=8). 'Anestrus' was defined as gilts that were culled because no behavioral estrus was noticed (Tummaruk *et al*., 2009) whereas other reasons not related to reproductive problems consisted of leg weakness or leg injury. The ovaries were separated from the genital tract and the stages of the estrous cycle evaluated. The presence of follicles, corpora lutea (CL) and corpora albicantia (CA) on both ovaries were used for classifying ovarian phases into the prepubertal, follicular and luteal phases. Briefly, ovaries with the appearance of numerous small follicles (diameter <5 mm) without CL or CA were judged as the 'prepubertal phase', ovaries with large follicles (diameter >5 mm) together with or without CA were designated as the 'follicular phase' while the 'luteal phase' was characterized by ovaries that contained most CL with or without small follicles and/or CA. In the follicular phase, the follicles at a diameter >5 mm were counted on the collected ovary. One random side of an ovary was immersed in 4% paraformaldehyde for performing investigation by histological technique and immunohistochemistry.

Immunohistochemical procedures and evaluation: To determine the AMH in growing follicles of gilt ovaries, immunohistochemistry was conducted according to Almeida *et al.,* (2018) with minor modifications. Briefly, the ovarian tissues were incised into 5 µm thickness and put on Super-Frost coated glass slides (Menzel-

Graser, Freiburg, Germany), heated in an incubator at 40°C for 4 h, deparaffinized in xylene and rehydrated through graded ethanol dilutions. The tissue sections were immersed in 0.01 M citrate buffer (pH 6.0) in a microwave oven at 750 Watt about 15 mins, endogenous peroxidase activity was blocked with 3% H_2O_2 in methanol solution at room temperature for 20 mins and non-specific background staining was reduced by incubation with normal horse serum (Vector Laboratories, Burlingame, USA). A monoclonal mouse anti-AMH antibody (B-11: sc-166752, Santa Cruz Biotechnology, CA, USA) diluted 1:100 was applied as the primary antibody. After the primary antibody addition, the histological sections were incubated overnight at 4°C in a humid chamber. Afterwards, the ovarian sections were incubated with secondary biotinylated horse anti-mouse antibody, followed by Avidin-Biotin Complex (ABC)-mouse reagent (Vectastain ABC kit, Vector Laboratories). The positive staining was accomplished using the 3, 3' diaminobenzidine (Immpact DAB kit, Vector laboratories) and counterstained with Mayer's hematoxylin. Finally, all tissue sections were mounted with gelatin-glycerine mixture. The ovaries from mature female dogs at the follicular phase were employed as positive controls, whereas the negative controls were completed by replacing the primary antibody with normal mouse IgG (Vector Laboratories). All tissue sections were analyzed under light microscopy (BX50) with a digital camera Micropublisher 5.0 (Qimage) and micrographs were carried out using Program Image Pro® Plus version 6.0 (Media Cybernatics). The immunostaining results were categorized as absent (–), weak (+), moderate (++) or strong (+++) based on the staining intensity as performed by former report in AMH immunoexpression (Rocha *et al*., 2016).

Follicular classification: The follicles in this experiment were categorized according to a previous report (Flaws *et al*., 1997) with minor adaptations. Concisely, the 'healthy follicle' was recognized as the follicle containing an intact oocyte (if the oocyte were visible), organized granulosa, theca internal layers and no pyknotic nuclei in follicular/granulosa cells whereas an 'atretic follicle' was determined as it included a degenerating oocyte (if the oocyte were noticed), disordered granulosa layer, shriveled follicular/granulosa cells and/or pyknotic nuclei. Follicles were scored as 'preantral follicles' if they contained an oocyte (if the oocyte were seen) and more than one layer of granulosa cells and a developing theca layer. Follicles that found at least one antrum appeared with a diameter smaller or larger than the oocyte diameter but the oocyte (if the oocyte were seen) was located more or less in the center of the follicle and judged as 'early antral follicles'. The 'large antral follicles' revealed the eccentric oocyte (if the oocyte were seen) that was surrounded by granulosa cells, a large antrum and the theca layer did not reveal the breakdown of the basement membrane between the granulosa and theca layer. Finally, the 'preovulatory follicles' confined the extensive folding of the theca interna and granulosa layers due to disintegration of the basement membrane detachment granulosa and theca layer.

Statistical analysis: All data was statistically examined using Statistical Analysis System software (SAS Institute, Cary, NC, USA). Mean and standard deviation (SD) were calculated and used for demonstrate data. The immunostaining intensity scores obtained from healthy follicles in the control and anestrus groups were analyzed using Kruskal-Wallis test and compared with the Wilcoxon rank sum test (NPAR1WAY procedure of SAS). A *P*-value of <0.05 was considered as statistically significant.

Results

Animal data: On average, the body weight at culling of control gilts and anestrus was 156.63±9.62 kg and 151.42±22.74 kg, respectively. Based on the ovarian status, two gilts were at the prepubertal phase and six gilts showed the follicular phase in the control group. In anestrus, two gilts were at the prepubertal phase, seven gilts the follicular phase and three animals were at the luteal phase. The numbers of follicles in the control gilts at diameter >5 mm (14.88±3.40 follicles) was significantly higher (P<0.05) compared to the anestrus gilts (6.75±1.91 follicles).

AMH immunolocalization in ovarian tissues: As expected, the strong intensity of AMH immunohistochemical staining was clearly detected in the granulosa cells of the canine preantral follicle which served as the positive control (Fig. 1a). The positive immunolocalization was detected as 'dark brown' staining in the cytoplasm of granulosa cells and was vague in the ooplasm of canine oocytes. In contrast, the AMH immunoreaction was absent in the negative control (porcine preantral follicle) through use of the normal mouse IgG instead of primary antibody (Fig. 1b).

In the present experiments, the healthy preantral, early antral, large antral and preovulatory follicles were observed and the intensity scores of AMH immunostaining was noticed in individual gilts of both groups. In the control group, moderate to strong positive immunostaining was detected in all types of growing follicles (Fig. 2). The pattern of AMH immunoexpression was heterogeneous and the intense staining slightly decreased in the granulosa layer of the preovulatory follicles (Fig. 2d). At higher magnifications, the characteristic of AMH immunoreaction was clearly seen as a brown color within the cytoplasm of granulosa cells (Fig. 3) and was not found in oocytes similar to the positive control. In atretic follicles, the AMH immunostaining was absent or of weak intensity in most granulosa cells (Fig. 4). In the anestrus group, all selected healthy follicles were considered and a moderate to weak AMH immunoreaction existed (Fig. 5). In the overall evaluation, however, intensity scores of AMH in preantral, small antral, large antral and preovulatory follicles of the anestrus group expressed significantly lower (P<0.05) compared with the same types of growing follicles in the control group (Fig. 6).

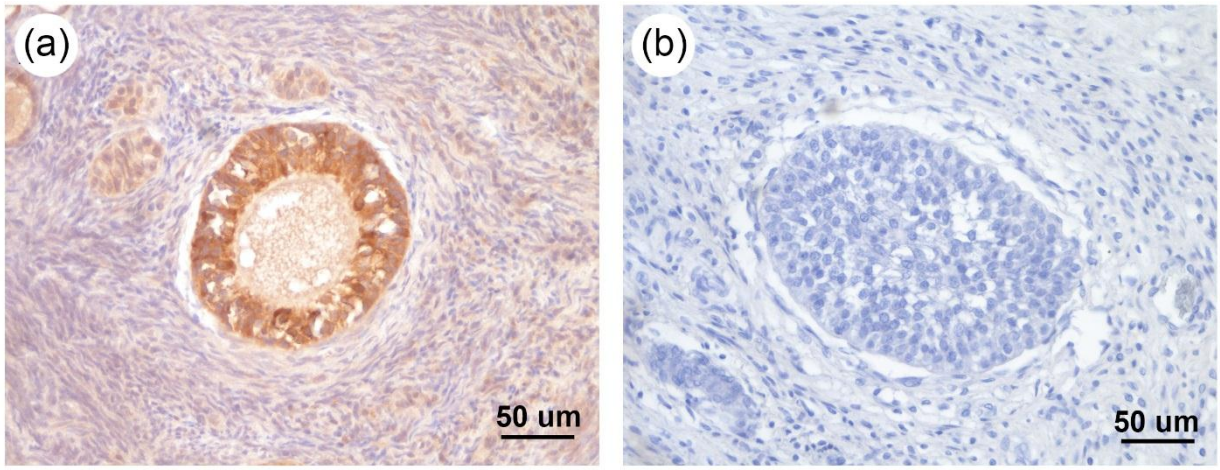

Figure 1 Positive and negative control sections for AMH immunohistochemistry. The preantral follicle of the canine ovary functioned as the positive control which showed brown staining in the cytoplasm of granulosa cells (a). Omission of the primary antibody and replacement by normal mouse IgG applied in porcine preantral follicle served as the negative control (b).

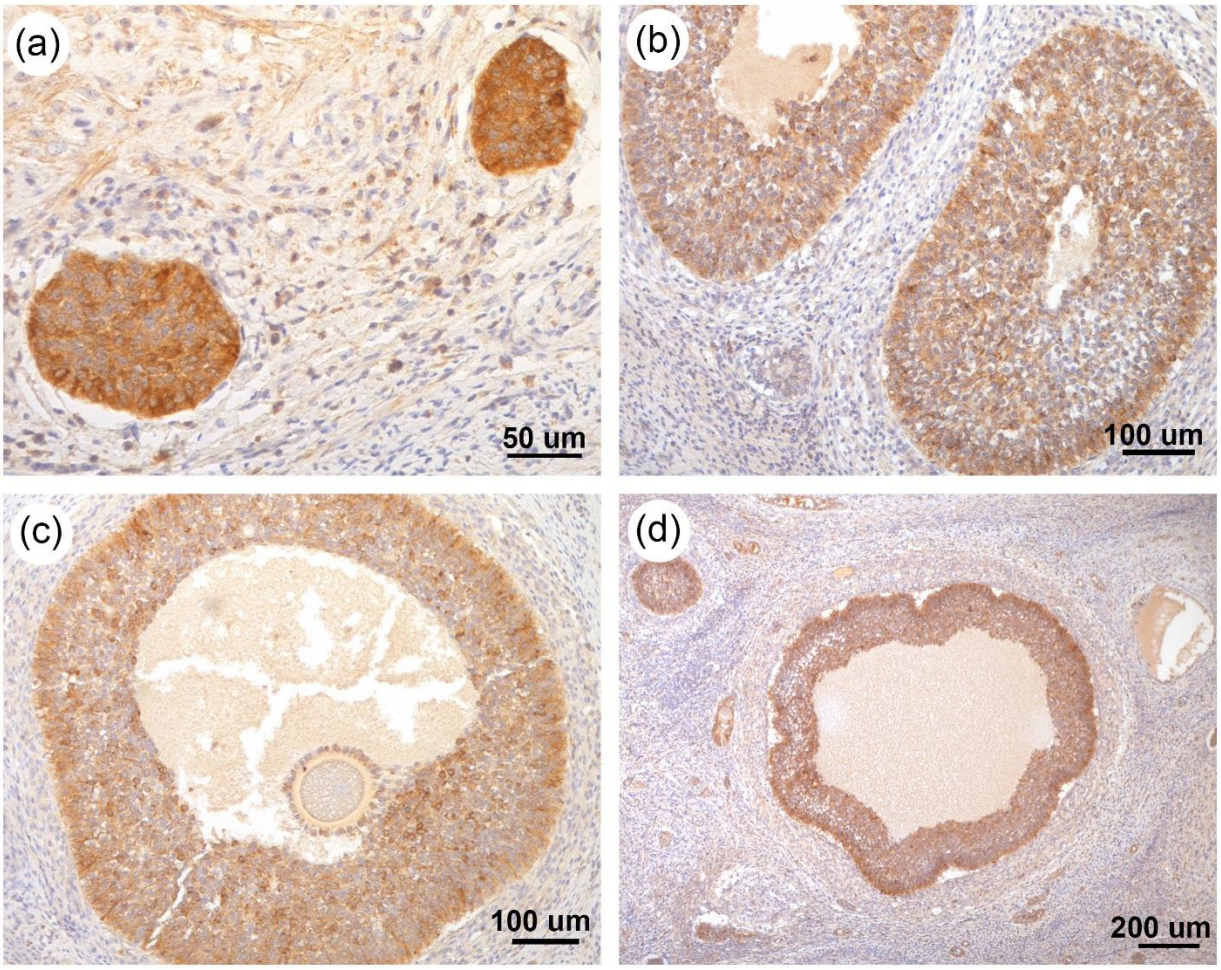

Figure 2 Representative photographs of strong AMH immunostaining of the preantral (a), early antral (b), large antral (c) and preovulatory (d) follicles in the ovaries of gilts culled due to other causes that were not related in reproductive problems. Notice the moderate to strong intensity occurring in the granulosa layer of the follicular growth.

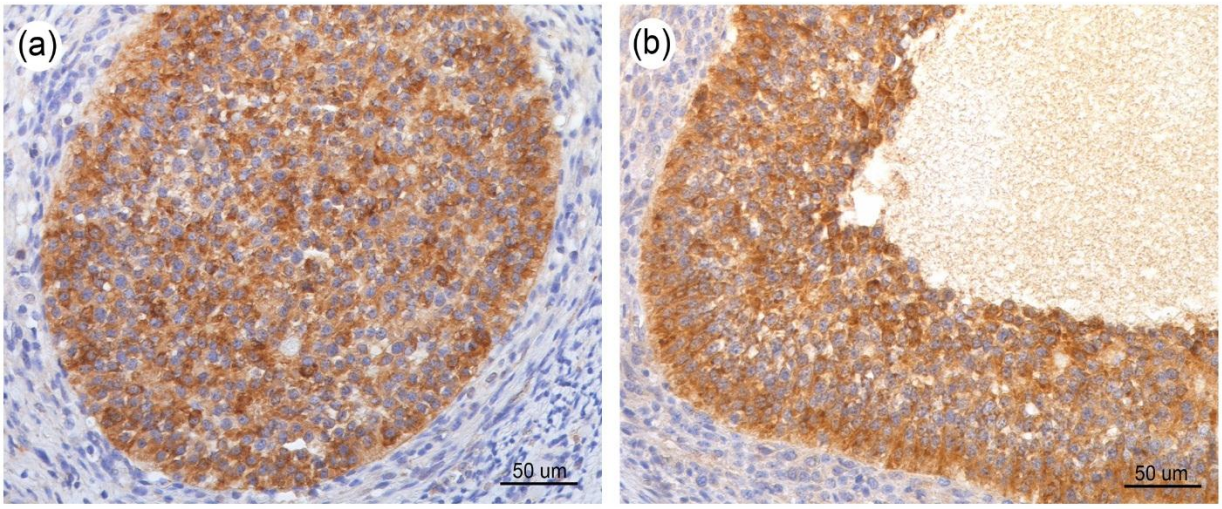

Figure 3 Representative photographs of AMH immunohistochemistry at higher magnifications of the preantral (a) and large antral (b) follicles of the gilts culled due to other causes that were not related to reproductive problems describing the brown immunostaining in the cytoplasm of granulosa cells.

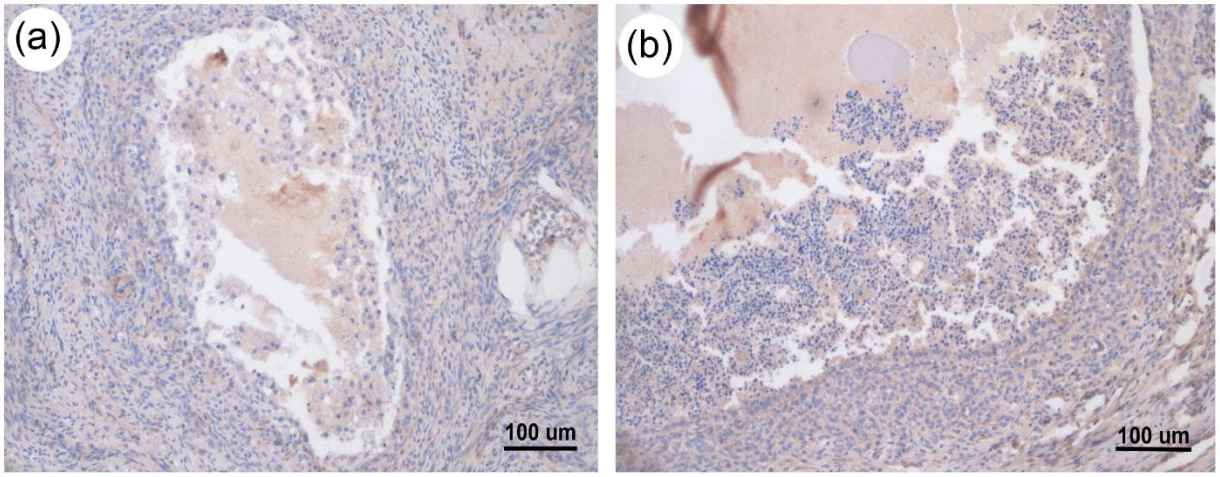

Figure 4 Light microscopic photographs of the atretic follicles at probably early antral (a) and large antral (b) follicular stages in the culled gilt ovaries.

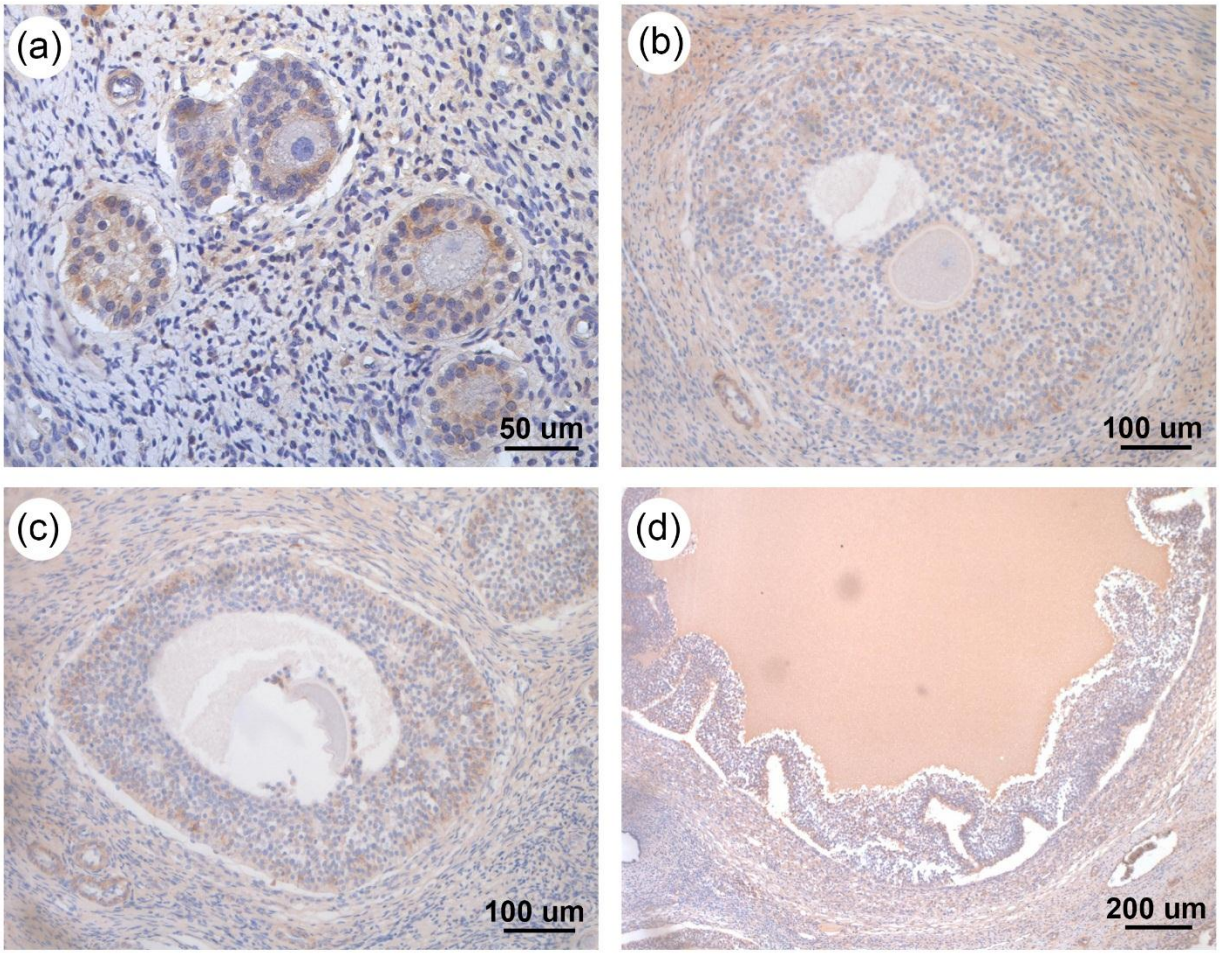

Figure 5 Representative photographs of weak AMH immunostaining of the preantral (a), early antral (b), large antral (c) and preovulatory (d) follicles in the ovaries of anestrus gilts.

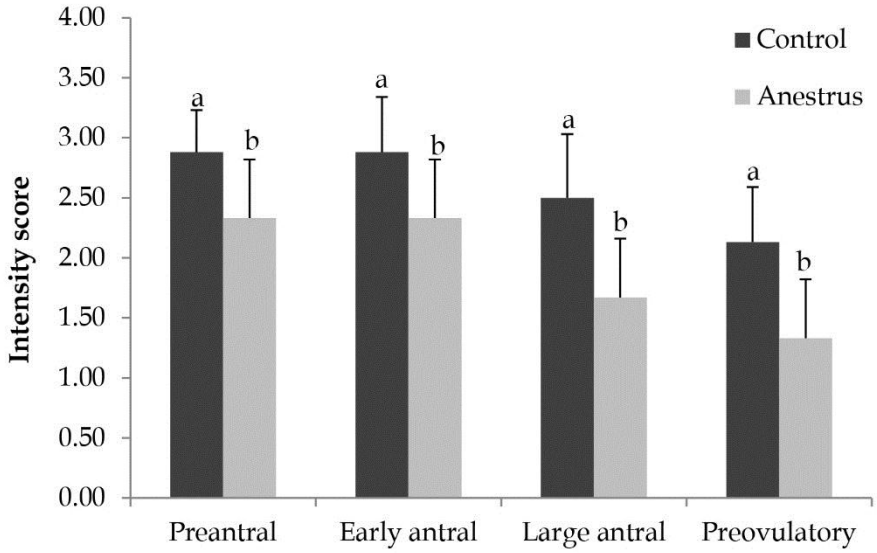

Figure 6 Intensity scores (mean ± SD) of AMH immunolocalization in the granulosa layers of the preantral, early antral, large antral and preovulatory follicles between the control group and anestrus group. [a,b] mean significant difference between groups with $P<0.05$.

Discussion

The cause of anestrus in Thailand needs to be clarified in culled gilts because the reproductive organs did not frequently display any pathological lesions. In this aspect, the reproductive tract, i.e. oviduct and uterus, of the anestrus gilts was observed by microscopic investigation and abnormalities were found in different ways as presented in previous studies (Teamsuwan *et al.*, 2010; Teh *et al.*, 2018; Tienthai and Sajjarengpong, 2007; Tienthai *et al.*, 2010).

Of course, the ovaries play a vital role in the reproductive process (e.g. producing oocytes and female steroid hormones), therefore the deformities that occur during follicular growth must be involved in reproductive failure in gilts. The present findings confirmed that a low intensity of AMH immunoexpression was detected in anestrus gilts indicating a reduced amount of AMH produced by granulosa cells of anestrus ovaries. Inadequate AMH might interrupt the physiological ovarian function and induce anestrus in replacement gilts.

With regard to the follicular stages, this experiment attempted to emphasize the four types of follicular stages composed of the preantral, early antral, large antral and preovulatory follicles of the culled gilts because these stages were obviously different and more easily observed than the primordial or primary follicles. In the present findings, AMH immunolocalization of both the control and anestrus groups was clearly exhibited in the granulosa cells of all selected types of 'healthy' follicles corresponding with the former reports in pigs (Almeida *et al*., 2018; Estienne *et al*., 2020). In the control pigs, the AMH intensity staining was no different between the types of ovarian follicles though the slightly heterogeneous nature of staining was seen in the granulosa layer of the preovulatory follicles. The earlier studies confirmed our results and indicated that the AMH immunoexpression in porcine ovaries was different from other species because the large antral and preovulatory follicles exhibited less intense staining than other types of growing follicles (Modi *et al*., 2006; Kevenaar *et al*., 2006; Rocha *et al*., 2016; Almeida *et al*., 2018). Additionally, AMH immunoreaction of atretic follicles in this study was very faint or non-detectable which is relevant to other reports in numerous species (Ueno *et al*., 1989; Juengel *et al*., 2002; Modi *et al*., 2006; Estienne *et al*., 2014) including pigs (Almeida *et al*., 2018). The present results explain that AMH is mainly produced by granulosa cells of 'healthy' growing follicles throughout the reproductive life of gilts. We also found that the intensity of staining of AMH immunoexpression in selected follicles of the anestrus gilts was significantly lower (P<0.05) than the control gilts. As the AMH antibody applied in this study was to detect the precursor and mature AMH protein, therefore, the faint intensity observed in the granulosa layer indicated a smaller amount of AMH produced by granulosa cells of the 'anestrus' gilts and may be associated with unsuitable ovarian functions.

As we know, AMH plays two important roles in female mammals, i.e. AMH is able to impede primordial follicular growth from the ovarian pool by avoiding premature exhaustion of the follicular reserves and it reduces the sensitivity of the pre-antral and antral follicles to follicular stimulating hormone (FSH) while controlling follicular growth (Durlinger et al., 2002; Dewailly *et al*., 2014). The appearance of strong or faint intensity AMH in preantral to early antral follicles confirmed that AMH is one of the growth regulatory factors for ovarian follicles and might be a more principal regulator of early follicle growth than FSH as hypothesized for rodents (Durlinger *et al*., 2001; Visser *et al*., 2007). Unquestionably, the variable AMH expression by immunohistochemical technique could involve the normal or abnormal physiological function of mammalian ovaries. For instance, the reduction of AMH immunoexpression in antral follicles was observed in women with premature ovarian failure syndrome (Méduri *et al*., 2007) and the faint intensity of AMH immunostaining was noticed throughout follicular growth in dairy cows suffering from cystic ovarian disease (Diaz *et al*., 2018). In the present study, the intensity staining of AMH immunoexpression in all follicular stages of anestrus gilts displayed significantly less (P<0.05) than the control groups indicating reduced production and function of granulosa cells. Therefore, the present results and the former reports about the aberration of AMH immunostaining suggest that decreased AMH production by granulosa cells can alter the mechanism of follicular recruitment and directly affect the ovarian functions. Interestingly, the serum AMH level was consistent with the quantitative analysis of AMH in the ovaries indicating that the AMH level in serum correlates to the number of primordial follicles and growing follicles (Jamil *et al*., 2016; Umer *et al*., 2019). Furthermore, several reports have recommended that circulating AMH is a good indicator of ovarian reserves and antral follicle populations in sheep (Torres-Rovira *et al*., 2014), mares (Claes *et al*., 2014) and cattle (Batista *et al*., 2014). Taken together, the AMH concentrations in serum demonstrated a positive interaction with the immunohistochemically strong positive antral follicles in bitches which are poly-ovulatory animal like pigs and the AMH levels were found to be lower in the anestrus phase of estrous cycle (Karakas Alkan *et al*., 2019). Up to date, a few studies in pigs have reported that lower plasma AMH concentrations were related to the low number of antral follicles in wild hybrid gilts (poor performance pigs) compared to Large White gilts (Tanihara *et al*., 2019) and, interestingly, the serum AMH levels were depicted to be significant lower in 'anestrus' gilts in Thailand (Am-In *et al*., 2020). Although serum AMH concentrations were not measured in the present study, it is possible that the lower circulation AMH level in these gilts arose from the smaller production of granulosa cells which is represented by weak immunostaining throughout follicular development. Possibly, less immunostaining of AMH found in follicular growth in anestrus gilts' ovaries might be associated with the inadequate serum AMH level resulting in disorder of the function of the ovaries.

In conclusion, this study revealed that AMH in normal pigs is detected by granulosa cells with constant intense immunoexpression from preantral to preovulatory follicles whereas the lower immunoreaction occurred in anestrus gilts whose reproductive organs rarely reveal pathological lesions at autopsy. These findings indicate the AMH as a member of TGF-ß might be regulated together with FSH to promote follicular reserve and survival via TGF-ß signaling pathways. The depletion of AMH in follicles which is relevant to the lower serum AMH levels in anestrus gilts in previous studies might have disrupted the ovarian physiological function causing anestrus in replacement gilts.

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