Livestock Science 150 (2012) 425–431

Contents lists available at SciVerse ScienceDirect

Livestock Science

journal homepage: www.elsevier.com/locate/livsci

Short communication

The use of proliferating cell nuclear antigen (PCNA) immuno-staining technique to determine number and type of follicles in the gilt ovary

Duangkamol Phoophitphong [a], Supradit Wangnaitham [b], Sayamon Srisuwatanasagul [c], Padet Tummaruk [a,*]

[a] Department of Obstetrics, Gynaecology and Reproduction, Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330, Thailand
[b] Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330, Thailand
[c] Department of Anatomy, Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330, Thailand

ARTICLE INFO

Article history:
Received 7 February 2012
Received in revised form
21 September 2012
Accepted 8 October 2012

Keywords:
Follicle
Gilt
Immunohistochemistry
Ovary

ABSTRACT

The present study determined the number and type of follicles in gilts ovarian tissue using the proliferating cell nuclear antigen (PCNA) immunohistochemical labelling technique and investigated the association between the number of follicles, gilt age, body weight, average daily gain, age at first observed oestrus, ovulation rate and weight of the ovaries. Ovarian tissues were obtained from 19 gilts aged 267.8 ± 19.2 days weighting 145.7 ± 11.8 kg. The tissues were incubated with mouse monoclonal anti-PCNA. The follicles were categorized as primordial, primary and growing follicles. PCNA immuno-staining enhanced the visualization of small follicles and the efficacy to distinguish primordial and primary follicles. The gilt ovarian tissue contained 19.8 ± 8.5 follicles/100 μm^2 (range 6.0–42.0). The numbers (and proportions) of primordial, primary and growing follicles per 100 μm^2 of the gilt ovarian tissue were 13.1 ± 6.9 (64.2%), 6.2 ± 3.3 (32.7%) and 0.5 ± 0.2 (3.1%) follicles, respectively. The number of primary follicles per 100 μm^2 of the gilt ovarian tissue positively correlated to body weight ($r=0.50$, $P=0.032$) but negatively correlated to age at first observed oestrus ($r=-0.54$, $P=0.015$). In conclusion, PCNA technique can be applied to quantify the precise number and distinguish the type of follicles in the ovarian tissue of porcine species. Gilts with a higher body weight and earlier age at first observed oestrus have a higher density of primary follicles in the ovarian tissue than those with a lower body weight and later age at first observed oestrus.

1. Introduction

The number and type of follicles in the ovary are indicators of folliculogenesis, which is associated with several hormones and growth factors (Myers et al., 2004). To quantify the number of ovarian follicles, ovarian tissue sections are conventionally performed on hematoxylin and eosin (H&E) tissue section. This method is time consuming and it is difficult to differentiate the type of small follicles (Bolon et al., 1997; Bucci et al., 1997). However, an immunohistochemical labelling of oocytes within the ovaries using an antibody against proliferating cell nuclear antigen (PCNA) has been reported in rats (Muskhelishvili et al., 2002; Oktay et al., 1995; Picut et al., 2008). The PCNA method enhances the visibility of primordial and primary follicles of the rat's ovary. Compared with H&E stained, the counting of ovarian follicles in PCNA-stained follicles had dramatically reduced variability from 11% to 0.2% and reduced the counting time

* Corresponding author. Tel.: +66 2 218 9644; fax: +66 2 255 0738.
E-mail address: Padet.t@chula.ac.th (P. Tummaruk).

1871-1413/$ - see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.livsci.2012.10.008

by 46% (Muskhelishvili et al., 2005). An earlier study of the PCNA method has also been done in pig ovaries (Tománek and Chrowska, 2006). However, the appearance of small follicles has not yet been clearly identified.

The immuno-staining of PCNA is depending on several factors, e.g., the number of proliferating cells in the tissue sample, the concentration of primary antibodies, the pretreatment of tissue with heat-induced epitope retrieval (HIER) technique and the type of fixative (Muskhelishvili et al., 2005). It has been demonstrated that the primordial follicle of bovine pre-culture ovarian tissue is not stained by PCNA if the tissue sample is fixed in Bouin's solution (Wandji et al., 1996). In rat formalin-fixed, paraffin-embedded ovaries, PCNA is strongly visualized when combination of HIER technique and a high concentration of primary antibody are used (Muskhelishvili et al., 2005). However, to our knowledge, the HIER technique for PCNA has not been done in pig ovarian tissue. The aims of the present study were to develop the optimal protocol for PCNA immunohistochemical labelling in pig ovarian tissue and to determine the number and type of the follicles in the gilt ovaries. In addition, the association between the number of follicles and some reproductive data (i.e., age and body weight of the gilt, average daily gain (ADG), age at first observed oestrus, ovulation rate and weight of the ovary) was investigated.

2. Materials and methods

2.1. Data, sample collection and tissue processing

The ovaries of 19 Landrace × Yorkshire crossbred gilts were selected from our previous study (Tummaruk et al., 2009). The genital organs collected from slaughterhouses were placed on ice and transported to the laboratory within 24 h of culling. Historical data for all gilts were collected, including the gilt identity, date of birth, date of first observed oestrus, date of culling and body weight at culling. Age at culling, age at first observed oestrus and ADG from birth to culling [ADG (g/day)=(body weight at culling − 1.5/age at culling) × 1000] were calculated. The ovaries were weighed using an electronic balance (BJ 210C, Precisa, Instruments Ltd., Switzerland). The appearance of the ovaries was used to assess the stage of the oestrous cycle. Corpora lutea (CL) were defined as coloured structures appearing on the ovaries which showed pink, tan or yellow, at a size of 7–12 mm in diameter. Corpora albicantia (CA) were defined as regressed and shrunken CL. Follicles were defined as transparent fluid-contained structures in the ovaries (Tummaruk et al., 2009). Only the ovaries that were classified as luteal phases were included in the present study (19 gilts). The luteal phase was characterized by ovaries containing CL with or without small follicles and/or CA. The ovulation rate was defined as the total number of CL from both ovaries. The ovaries were fixed in 10% neutral-buffered formalin for 24–48 h, processed by an automatic tissue processor (Tissue-Tek VIP 5 Jr., Sakura, Tokyo, Japan) and embedded in paraffin block (Tissue-Tek TEC, Sakura, Tokyo, Japan). The paraffin embeddings were cut into 5 μm thickness using microtome (Shandon, Anglia scientific instrument Ltd., Cambridge, UK). For each ovarian tissue, two sections were cut serially and each section was placed on a separate slide, resulting in two sets with nearly identical ovarian sections. One set of the section was stained with H&E, while the other was stained by PCNA immunohistochemistry.

2.2. Immunohistochemistry

PCNA immuno-staining technique has been modified after previous studies in the rat's ovarian tissue (Muskhelishvili et al., 2005; Picut et al., 2008) and in mammary tumours of cats (Taweechart et al., 2004). Briefly, the pig ovarian tissues were deparaffinized and placed in an antigen retrieval solution (0.01 M citrate buffer, pH 6.0) for 10 min in a microwave oven at 600 W. Endogenous peroxidase was blocked using freshly prepared 3% hydrogen peroxide for 10 min at room temperature. Nonspecific staining was blocked with 1% bovine serum albumin for 30 min at 37 °C. The slides were incubated with mouse monoclonal anti-PCNA (clone PC10, DAKO, Carpinteria, CA, USA) as a primary antibody at a dilution of 1:200 overnight at 4 °C. After incubation with the primary antibody, the sections were incubated with DAKO EnVision™ reagent for 45 min at room temperature. Staining was developed with 3,3′-Diaminobenzidine tetrahydrochloride hydrate (DAB, Sigma, Germany) for 3 min at room temperature. The sections were counterstained with Mayer's hematoxylin for 1 min, dehydrated through graded series of ethanol, placed into xylene and mounted with mounting media. During the steps of the staining procedure, slides were washed three times in phosphate-buffered saline solution (PBS) for 5 min each time. For negative control, PBS was used instead of the primary antibody.

2.3. Histological examination

Histological examination for PCNA immuno-staining was carried out in one ovarian tissue section per gilt. The ovarian follicles were quantified under light microscope with a magnification of 40× for determining growing follicles and 100× for determining primordial and primary follicles (B × 40, Olympus, Japan). For each section, 10 microscopic fields were arbitrarily selected for investigation and were counted in 25 squares of ocular micrometre that corresponded to 156.25 μm^2 (40×) and 25.0 μm^2 (100×) of real tissue area. Therefore, the total tissue area used to determine the number of primordial follicles, primary follicles and growing follicles was 250 μm^2, 250 μm^2 and 1562.5 μm^2, respectively. The quantity of follicles in the selected area was determined. Changing the location of the ocular micrometre was done across the entire area in a non-overlapping manner. Histological examination of the samples was accomplished by only one person (D. Phoophitphong) who was unaware of the identity of the gilts. In the present study, only the numbers of follicles with an intact oocyte was counted to minimize the possibility of including degenerated oocytes. The number of follicles was expressed as the total number of follicles per 100 μm^2 of the tissue section (see below).