Original Article

Expression of the Progesterone Receptor in the Thai Swamp Buffalo Oviduct at the Follicular and Luteal Phases

Paisan Tienthai[1*] Kriengyot Sajjarengpong[1] Mongkol Techakumphu[2]

Abstract

The objective of this study was to investigate the presence and distribution of the progesterone receptor (PR) in the Thai swamp buffalo oviduct at the follicular (n=10) and luteal phases (n=12). Reproductive tracts from mature female Thai swamp buffaloes were collected from a local slaughterhouse. Both ovaries were examined for the phase of estrous cycle and the oviducts were separated into the uterotubal junction (UTJ), the isthmus, the ampulla and the infundibulum. Tissue samples were subjected to immunohistochemical staining to assess the localization of PR in the epithelium, subepithelial connective tissue and smooth muscle. A positive nuclear immunolabeling of PR was detected in all three compartments of the buffalo oviduct and the average staining was influenced by the phase of the estrous cycle. There was more intensity ($p<0.05$) and a larger proportion ($p<0.05$) of PR immunostaining during the follicular phase than that during the luteal phase in the UTJ and the isthmus. However, the tendency of PR was also greater in the ampulla and infundibulum at the follicular phase. Noticeably, nuclear PR staining was more commonly detected in the secretory cells than in the ciliated cells. In conclusion, the results of this study suggest that PR expression was clearly detected in Thai swamp buffalo oviduct and varied according to the oviductal segment as well as with the marked changes in the period of estrous cycle.

Keywords : estrous cycle, oviduct, progesterone receptor, Thai swamp buffalo

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บทคัดย่อ

การแสดงออกของตัวรับฮอร์โมนโปรเจสเตอโรนในท่อนำไข่กระบือปลักไทยระยะฟอลลิคูลาร์และระยะลูเทียล

ไพศาล เทียนไทย[1*] เกรียงยศ สัจจเจริญพงษ์[1] มงคล เตชะกำพุ[2]

การศึกษานี้ มีวัตถุประสงค์เพื่อตรวจสอบการปรากฏและการกระจายของตัวรับฮอร์โมนโปรเจสเตอโรนในท่อนำไข่ส่วนต่างๆ ของกระบือปลักไทยในระยะฟอลลิคูลาร์และระยะลูเทียล เก็บตัวอย่างท่อทางเดินสืบพันธุ์ของกระบือปลักไทยเพศเมียจากโรงฆ่าสัตว์ท้องถิ่น ตัดรังไข่ทั้งสองข้างเพื่อศึกษาลักษณะโดยทั่วไปเพื่อกำหนดระยะของวงรอบการเป็นสัด แยกท่อนำไข่ออกจากทางเดินสืบพันธุ์และตัดแบ่งออกเป็นส่วนต่างๆ ประกอบด้วย ส่วนรอยต่อระหว่างอิสธ์มัสกับปีกมดลูก อิสธ์มัส แอมพูลลา และอินฟันดิบูลัม ศึกษาการแสดงออกของตัวรับโปรเจสเตอโรนในชั้นเยื่อบุ ชั้นเนื้อเยื่อเกี่ยวพันใต้เยื่อบุ และชั้นกล้ามเนื้อเรียบของท่อนำไข่กระบือปลักไทยแต่ละส่วนด้วยวิธีอิมมูนโนฮิสโตเคมี ผลการทดลองพบว่า มีการย้อมติดสีของตัวรับโปรเจสเตอโรนปรากฏขึ้นในนิวเคลียสของเซลล์ที่พบในชั้นต่างๆ ทั้ง 3 ชั้นของท่อนำไข่ ซึ่งค่าเฉลี่ยของการย้อมติดสีมีการแปรปรวนไปตามวงรอบการเป็นสัด โดยความเข้มและสัดส่วนของการย้อมติดสีบวกเพิ่มมากขึ้นอย่างมีนัยสำคัญทางสถิติในส่วนรอยต่อระหว่างอิสธ์มัสกับปีกมดลูกและส่วนอิสธ์มัสระยะฟอลลิคูลาร์ อย่างไรก็ตาม ผลการย้อมติดสีบวกในท่อนำไข่ส่วนแอมพูลลาและอินฟันดิบูลัมมีแนวโน้มสูงขึ้นในระยะฟอลลิคูลาร์เช่นเดียวกัน และเป็นที่น่าสังเกตว่า การย้อมติดสีของตัวรับฮอร์โมนโปรเจสเตอโรนในชั้นเยื่อบุนั้นปรากฏให้เห็นชัดเจนในนิวเคลียสของเซลล์คัดหลั่งเมื่อเปรียบเทียบกับเซลล์ที่มีซีเลีย จากการศึกษานี้สรุปได้ว่า การแสดงออกของตัวรับฮอร์โมนโปรเจสเตอโรนสามารถตรวจพบได้ในชั้นต่างๆ ของท่อนำไข่กระบือปลักไทย ซึ่งมีการเปลี่ยนแปลงไปตามหน้าที่ของท่อนำไข่แต่ละส่วนและระยะต่างๆ ของวงรอบการเป็นสัด

คำสำคัญ : วงรอบการเป็นสัด ท่อนำไข่ ตัวรับโปรเจสเตอโรน กระบือปลักไทย

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Introduction

The oviduct provides a suitable microenvironment for critical biological events occurring during gamete transport, sperm reservoir, sperm capacitation, oocyte maturation, fertilization and early embryonic development before the implantation of the embryo in the uterus, and these regulations are involved in the surface epithelium, connective tissue in the subepithelium and the muscular layer of the oviduct (Ellington, 1991; Hunter, 2003).

Similar to other mammalian oviducts, the epithelium of the Thai swamp buffalo oviduct is composed of ciliated and secretory cells in which morphological changes are demonstrated markedly during the estrous cycle (Tienthai et al., 2008). The cilia of ciliated cells actively involved in the transport of oocytes increase in the infundibulum and the ampulla at the follicular phase and decrease in both segments during the luteal phase (Abe and Oikawa, 1993[a]; Abe et al., 1993). However, the secretory cells which are important in the production of specific nutrients, compound proteins and glycoproteins to form oviductal fluid (Leese et al., 2001), have been reported to show more activity in secretion in the caudal part of the ruminant oviduct, i.e. the uterotubal junction (UTJ) and the isthmus, at the follicular phase than that during the luteal phase (Abe and Oikawa, 1993[a]; Bergqvist et al., 2005; Tienthai et al., 2008). Several previous studies have indicated that changes of epithelial cells and other compartments of the oviduct correspond with the levels of estrogens and progesterone in the circulating plasma throughout the estrous cycle (Brenner

et al., 1974; Verhage et al., 1979; Abe and Oikawa, 1993[b]; Steinhauer et al., 2004). The proliferation of surface epithelium cells and the differentiation of secretory cells are regulated under the influence of estrogens (Abe and Oikawa, 1993[b]), whereas progesterone functions generally antagonistically to the estrogen-mediated effect described above (Steinhauer et al., 2004). Absolutely, some of these morphological changes also take place in the Thai swamp buffalo oviduct during follicular and luteal phases in which the phase of estrous cycle is only observed by the characteristics of both buffalo ovaries (Tienthai et al., 2008).

The diverse biological actions of progesterone are believed to be conveyed mostly through interaction with transcription-regulating nuclear progesterone receptors (PR), which are members of the steroid/nuclear receptor suprafamily (Tsai and O'Malley, 1994; Brosens et al., 2004). A better understanding of the function of the swamp buffalo oviduct in ovarian activity, particularly in the physiological conditions, might be clarified by the appearance of female hormonal receptors in the oviduct during periods of the estrous cycle. Although the immunolocalization of PR in the oviduct has been reported in ruminants including sheep (Garcia-Palencia et al., 2007), heifers (Valle et al., 2007) and cows (Ulbrich et al., 2003), the PR in the Thai swamp buffalo oviduct has not yet been observed. The objective of the present work was, therefore, to investigate the localization of PR in different segments, i.e. UTJ, isthmus, ampulla and infundibulum, of Thai swamp buffalo oviducts during the follicular and luteal phases.

Materials and Methods

Animals and tissue collection: Female genital organs were collected from mature Thai swamp buffalo cows (aged 2-8 years, n = 22) at the local abattoir and immediately put in a cool container at ~4°C for 30-45 min until being processed in the laboratory. The reproductive organs were investigated for general normality. The classification into two phases of the estrous phases was done by direct observation of the dominant follicles and corpus luteum in both ovaries as previously described for the buffalo ovaries (Ali et al. 2003) and later adopted for Thai swamp buffalo (Tienthai et al., 2008). The features of both ovaries during estrus (Fig. 1A) were classified as the follicular phase (n=10), whereas the ovaries with a full-growth of corpus luteum showing late diestrus (Fig. 1B) were grouped as the luteal phase (n=12). The swamp buffalo oviducts were then cut into four different segments composed of UTJ, isthmus, ampulla and infundibulum for immunohistochemical analysis.

Tissue preparations and immunohistochemistry: Tissue samples from the UTJ, isthmus, ampulla and infundibulum at both follicular and luteal phases were fixed in 10% neutral buffered formalin and then

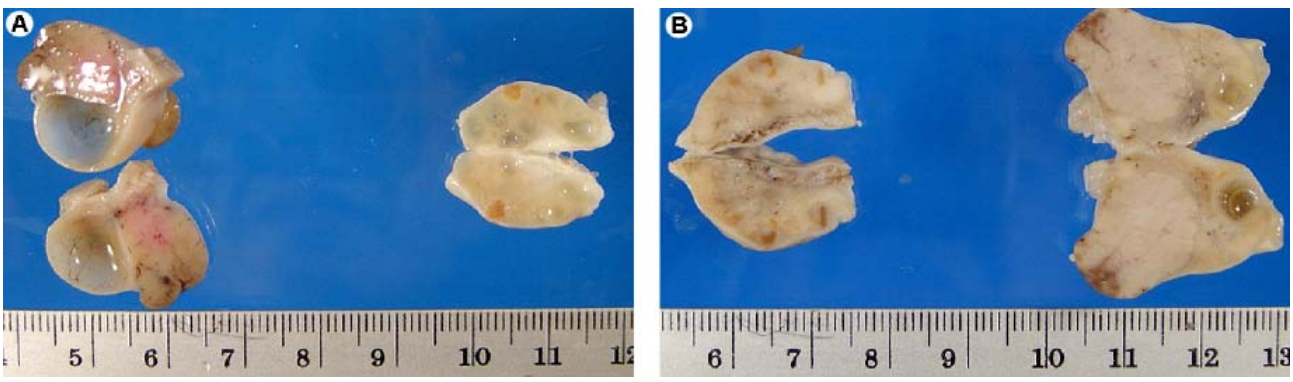

Fig. 1 Longitudinal sections of Thai swamp buffalo ovaries collected from the local abattoir showing the ovarian characteristics of the follicular (A) and luteal (B) phases

embedded in paraffin wax. Thin paraffin sections of 5-μm thickness were cut and mounted on SuperFrost slides (Menzel-Glaser, Freiburg, Germany). A standard immunohistochemical technique (avidin-biotin-peroxidase) used to detect the appearance of PR isoform B staining was described earlier by Bage et al. (2002). Briefly, the tissue sections were deparaffinized, rehydrated and pretreated in a microwave oven at 700 W, in 0.01 M sodium citrate buffer (pH 6.0) for antigen retrieval. After washing in buffer (0.1 M phosphate-buffered saline (PBS), pH 7.6), non-specific endogenous peroxidase activity was reduced by treatment with 3% H_2O_2 in methanol for 10 min at room temperature. The sections were exposed to a non-immunoblock using diluted normal horse serum (Vectastain; Vector Laboratories, Burlingame, CA, USA) in PBS for 30 min at room temperature. Tissue sections were thereafter incubated with the primary antibody. A monoclonal mouse anti-chicken PR isoform B antibody (MAI-411) was purchased from Affinity Bioreagents, Inc. (Golden, CO, USA), diluted 1:250 in PBS and incubated in sections at 4°C overnight. Normal bovine uterine horn known to express PR isoform B was served as positive controls, whereas the negative controls were obtained by replacing the primary antibody with mouse IgG at an equivalent concentration. After primary antibody binding, the sections were washed in PBS and incubated with a secondary antibody. A biotinylated horse anti-mouse IgG (Vector Laboratories) diluted in PBS was used for 45 min at room temperature. The tissue samples were then incubated with horseradish peroxidase-avidin-biotin complex (Vectastain ABC Elite; Vector Laboratories) for 60 min at room temperature. The PR immunoreactivity was visualized using freshly prepared diaminobenzidine (DAB kit; Vector Laboratories) and H_2O_2 in distilled water. All sections were counterstained with hematoxylin and mounted in glycerol gelatin.

Scoring of immunoreactive stained cells: Stained sections were investigated using a light microscope (BX50, Olympus, Tokyo, Japan) equipped with a digital camera (ImagePro6, Tokyo, Japan). The evaluation of results was performed in different tissue compartments of the buffalo oviducts comprising the layers of surface epithelium, subepithelial connective tissue and smooth muscle. Examinations of PR immunostaining cells were done by blind preparation and the same person. The intensity of PR immunoreactive cells was classified into three different levels as follows: weak, 1; moderate, 2 and strong, 3. Since not all cells were stained positively in the three compartments of oviduct, the proportion of positive to negative cells was estimated for these tissues into four different levels (marked 1-4): low proportion (<30% of positive cells, 1); moderate proportion (30-60% of positive cells, 2); high proportion (>60-90% of positive cells, 3) and almost all cells positive (>90% of positive cells, 4).

Statistical analyses: Data were analyzed using the SAS statistical package (version 8.0, SAS Institute, Inc., 1998, Cary, NC, USA). The normal distribution of residuals from the statistical models was tested using the UNIVARIATE procedure option NORMAL. Differences in the mean numbers of immune cells and cell heights were tested using analysis of variance (Proc MIXED). The statistical model included the fixed effects of stage (follicular and luteal) and segment (UTJ, isthmus, ampulla, and infundibulum); the interaction between stage and segment; and the random effect of buffaloes nested within stage. Bonferroni *t*-test was used to compare least-square means between groups when overall significance for that was found and p value ≤ 0.05 was considered statistically significant.

Results

The immunohistochemical examination with the specific PR monoclonal antibody allowed us to visualize progesterone target cells in the Thai swamp buffalo oviduct by mean of the detection of its receptor. The PR immunoreactive staining was mainly shown in the nuclei of the epithelial, subepithelial and smooth muscle cells of the oviduct as presented in Figure 2. The immunostaining

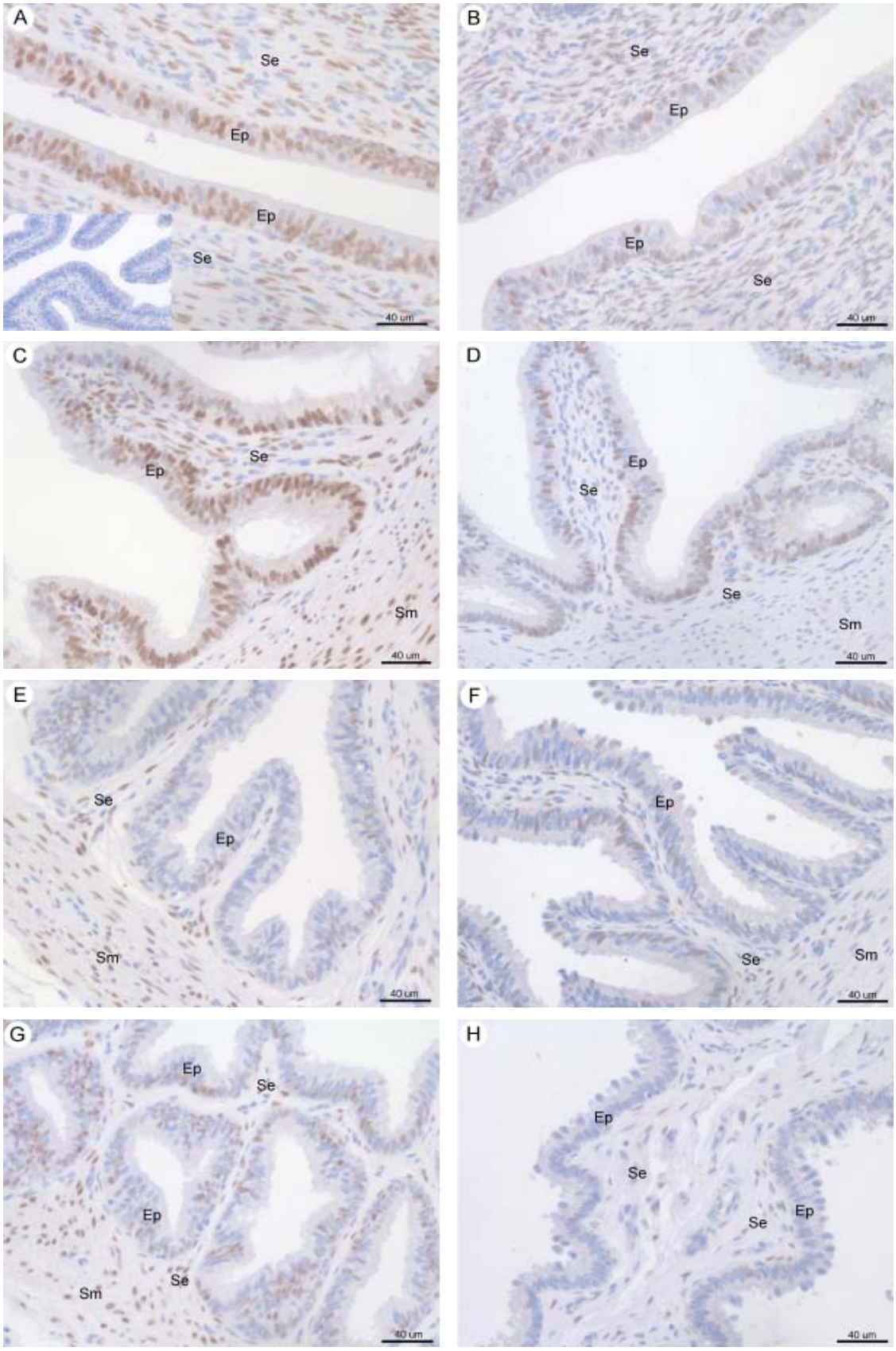

Fig. 2 Immunohistochemical demonstration of PR in different compartments of the Thai swamp buffalo oviduct at the follicular phase (A, C, E, G) and the luteal phase (B, D, F, H) of the estrous cycle. The nuclear PR positive cells were stained brown in the epithelium (Ep), subepithelial connective tissue (Se) and muscular layer (Sm) of UTJ (A, B), isthmus (C, D), ampulla (E, F) and infundibulum (G, H) of the oviduct.

for PR varied in all compartments of each oviductal segment according to the phases of the estrous cycle. Variations were observed regarding the intensity of positive staining and the proportion of positive/negative nuclei in the surface epithelium, subepithelial connective tissue and smooth muscle as described in Figures 3-5.

During follicular phase, the immunohistochemical localization for PR in the nuclei of epithelial cells showed intense staining in all segments of the buffalo oviducts (Fig. 2A, C, E, G). The intensity and proportion of PR immunostaining in the epithelial cells of the UTJ and isthmus were significantly greater (p<0.05) during the

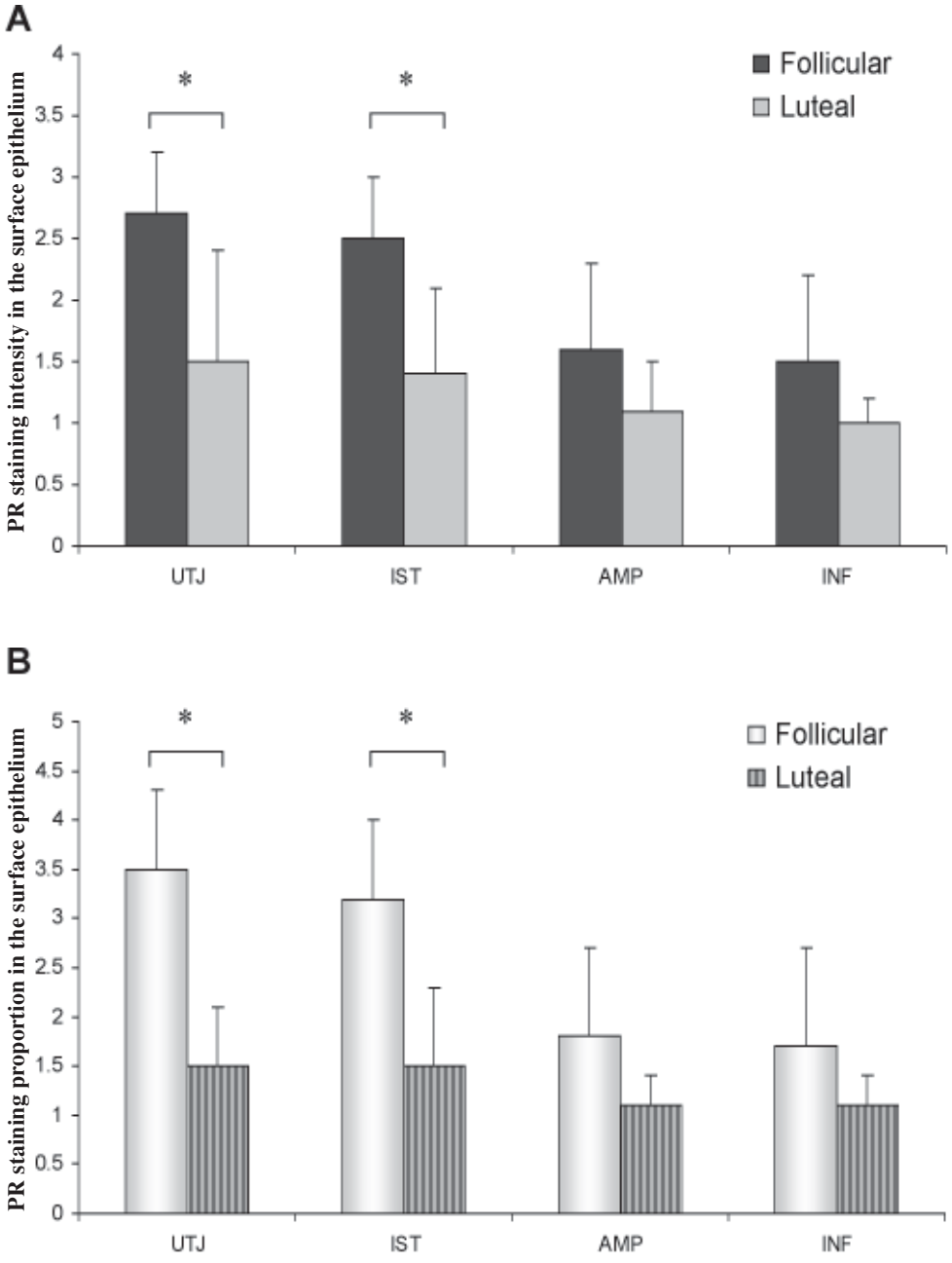

Fig. 3 Intensity (A) and proportion (B) scores of PR immunolocalization in the surface epithelium of the uterotubal junction (UTJ), the isthmus (IST), the ampulla (AMP) and the infundibulum (INF) of the Thai swamp buffalo oviduct at the follicular and the luteal phases. Values are presented as means ± SD.* means significant difference between follicular and luteal phases with p<0.05.

follicular phase than those in the luteal phase (Fig. 3A, B), while the PR immunolabeling of the epithelial cells in the ampulla and infundibulum was not different at either phase. In addition, nuclear PR immunostaining was commonly found in the secretory cells of the Thai swamp buffalo oviduct (Fig. 6A, B). The omission of the PR antibody revealed no staining in all three compartments of the buffalo oviduct (inset in Fig. 2A).

In the subepithelial connective tissue, the PR l ocalization was positive in the nuclei of connective tissue cells but not all cells (Fig. 2). The greater intensity and the higher proportion of cells with PR immunostaining were

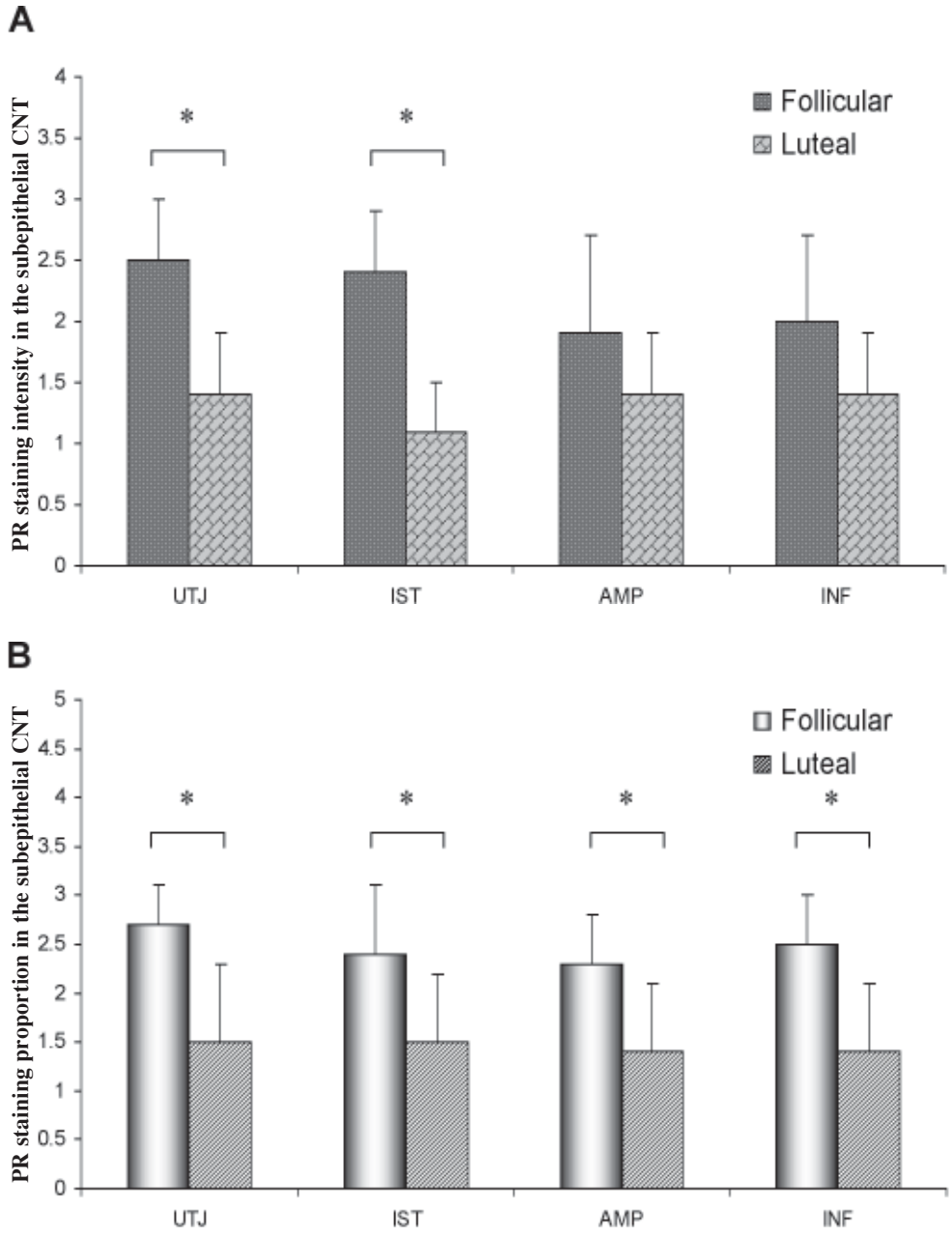

Fig. 4 Intensity (A) and proportion (B) scores of PR immunolocalization in the subepithelial connective tissue of the uterotubal junction (UTJ), the isthmus (IST), the ampulla (AMP) and the infundibulum (INF) of the Thai swamp buffalo oviduct at the follicular and luteal phases. Values are presented as means ± SD.* means significant difference between follicular and luteal phases with $p<0.05$.

significantly (p<0.05) found in the UTJ and isthmus at the follicular phase than those in the luteal phase. In addition, the immunoreactive staining in the subepithelial connective tissue cells of the ampulla and infundibulum tended to be high during the follicular phase (Fig. 4A, B).

Similar to the subepithelial layer, the smooth muscle nuclei, but not all nuclei, in the smooth muscle compartment of the buffalo oviduct reacted to PR immunostaining (Fig. 2) and the highest intensity proportion was also detected in the UTJ and isthmus at the follicular phase (Fig. 5A, B).

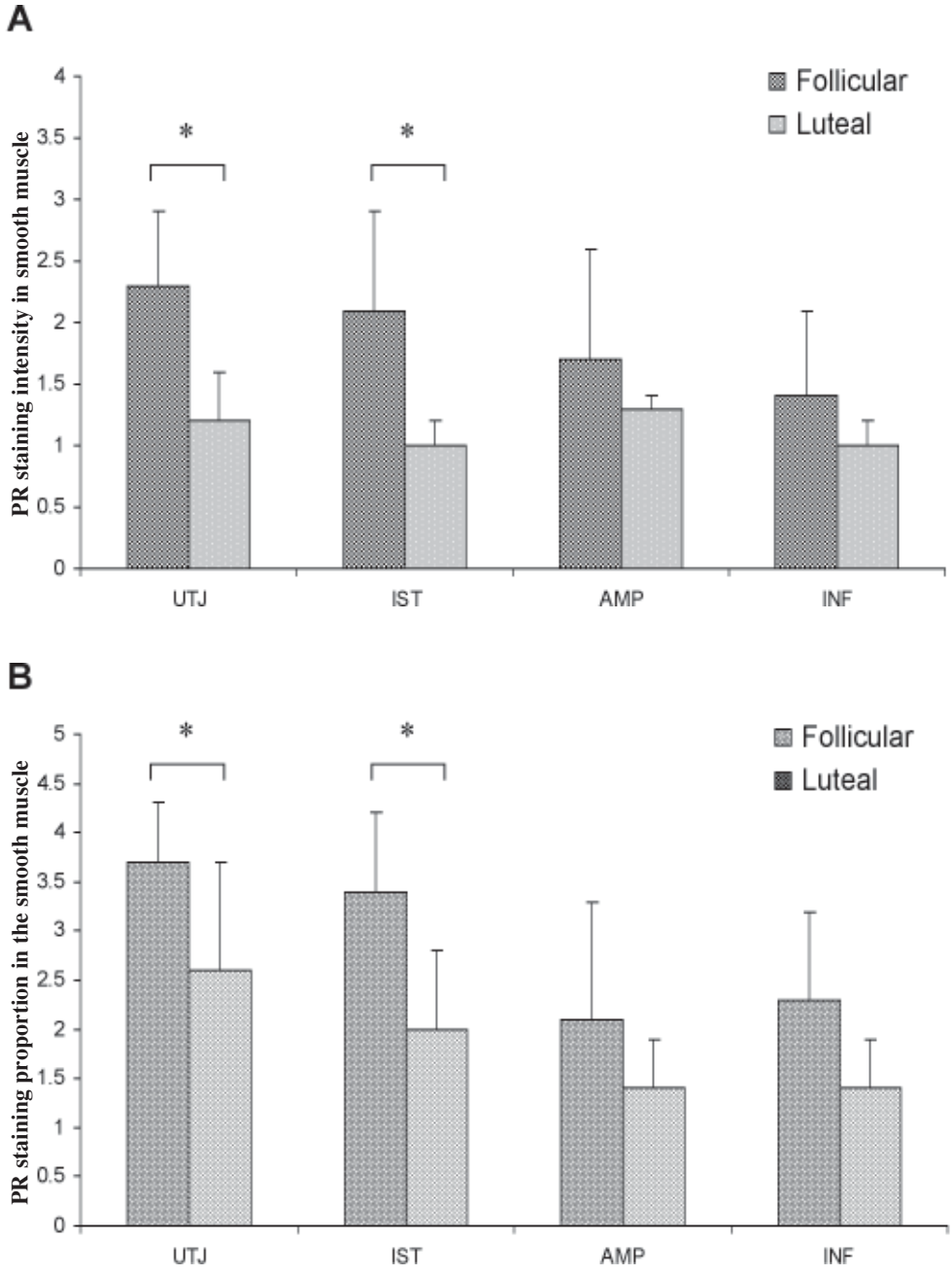

Fig. 5 Intensity (A) and proportion (B) scores of PR immunolocalization in the smooth muscular layer of the uterotubal junction (UTJ), the isthmus (IST), the ampulla (AMP) and the infundibulum (INF) of the Thai swamp buffalo oviduct at the follicular and luteal phases. Values are presented as means ± SD.* means significant difference between follicular and luteal phases with p<0.05.

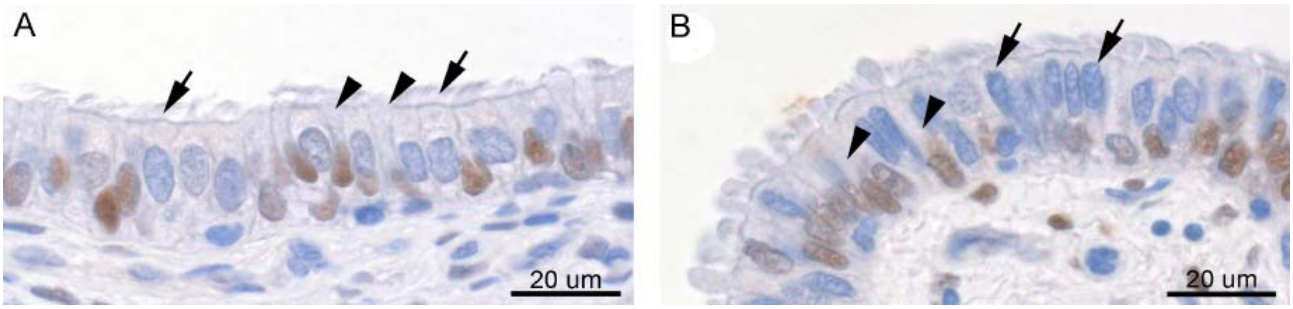

Fig. 6 Photomicrographs of PR immunolocalization in the isthmus (A) and the ampulla (B) of the Thai swamp buffalo oviduct at the follicular phase. Note the intensity of PR staining in the nucleus of secretory cells (arrowheads) compared with the negative nuclear PR staining in the ciliated cells (arrows).

Discussion

The present study demonstrated that the PR was detectable in the nucleus of surface epithelial cells, subepithelial connective tissue cells and smooth muscle cells of all portions along the oviduct of Thai swamp buffalo, which is in agreement with the earlier reports in cows (Ulbrich et al., 2003), heifers (Valle et al., 2007) and sheep (Garcia-Palencia et al., 2007). Interestingly, the patterns of PR immunostaining were demonstrated to be of significantly higher intensity and proportion ($p<0.05$) in all tissue layers of UTJ and isthmus at the follicular phase than the luteal phase. However, the PR had a tendency to appear clearly in the different compartments of ampulla and infundibulum at the follicular phase as well. These findings correlate to that reported in heifers (Valle et al. 2007) in which a reduction of staining intensity occurred from the UTJ and isthmus towards the infundibulum and the greatest intensity of PR staining in the oviduct always appeared during the estrus phase (i.e. the follicular phase). It is known that plasma estrogen levels in bovines are found to be highest during the follicular phase, whereas progesterone is dominant during the mid and late-luteal phase (Meyer et al., 1990). Incontrovertibly, the female reproductive organs including the uterus and oviduct are considered to be under the influence of these hormones which are mediated through their specific nuclear receptors to be able to exert their function and effects (Wijayagunawardane et al., 1998). In the uterus, studies in several species have reported that the highest of PR staining was found in most compartments during estrus (Stanchev et al., 1990; Geisert et al., 1994; Robinson et al., 2001; Ing and Tornesi, 1997; Bianchi et al., 2007) indicating that the estrogen up-regulates the PR whereas progesterone down-regulates its own receptor. Similar results have been observed in the mouse (Shao et al., 2006) that a time-dependent treatment *in vitro* with progesterone suppressed the expression of PR protein, whereas estradiol had a stimulatory effect on the expression of PR. Therefore, the present data may indicate that the PR in the Thai swamp buffalo oviduct could be up-regulated by the increase of plasma estrogen levels by the changes of dominant follicle and corpus luteum in both ovaries and that PR might be involved in the specific reproductive processes occurring in different parts of oviduct, particularly at the follicular phase. Nevertheless, PR immunolocalization in the Thai swamp buffalo oviduct together with the levels of female steroid hormones, ovarian status and the detection of PR mRNA throughout the estrous cycle have to be undertaken in the further study.

Concerning the PR localization in the oviductal epithelial cells, the results showed that the PR staining was commonly found in the secretory cells (Fig. 6A, B) in all segments of the Thai swamp buffalo oviduct. Several studies have reported that progesterone influences the morphological changes of the oviductal epithelium. For example, a decrease in the total number of ciliated cells, a decrease in the epithelial cell height (Abe and Oikawa, 1993[a]; Abe et al., 1993; Tienthai et al., 2008), the

dedifferentiation and regression of epithelial cells (Sawyer et al., 1984; Steinhauer et al., 2004) appear to be a consequence of the cellular and nuclear extrusion of secretory cells from the epithelium (Rumery et al., 1978; Odor et al., 1980). The morphological processes above were found in the ampulla and infundibulum during the luteal phase in cows (Abe and Oikawa, 1993[a]), goats (Abe et al., 1993) and swamp buffaloes (Tienthai et al., 2008) as also depicted in the present study (Fig. 2F, H). Although the PR was not significantly presented in the ampulla and infundibulum in this study (tending to be higher at follicular phase), it is possible that the PR staining found in the ampulla and infundibulum of the Thai swamp buffalo oviduct might be involved in the dedifferentiation and regression of secretory cells. The most important finding of our results indicated that the highest intensity and proportion of PR staining was detected in the secretory epithelial cells at the follicular phase of isthmus and UTJ, indicating that these parts of the buffalo oviduct were the main target of the PR which is stimulated by estrogens. Generally, the caudal isthmus and UTJ of mammalian oviduct are implicated in events of sperm transport, storage and capacitation that require the preservation of the motility, viability and fertilizing ability of spermatozoa before ovulation occurs (Pollard et al., 1991; Lefebvre et al., 1995). In previous studies, the morphological features such as the changes of epithelial cell height, cellular differentiation and protrusion as well as the proportion of secretory and ciliated cells in the isthmus and UTJ did not show any differences in the cyclic changes in cows (Abe and Oikawa, 1993[a]), goats (Abe et al., 1993) and Thai swamp buffaloes (Tienthai et al., 2008). However, the secretory cells in these regions clearly showed the secretory activity by staining with periodic acid-Schiff at follicular phase (Tienthai et al., 2008) and the secretion containing glycoproteins and mucopolysaccharides in these parts was important in the formation of the sperm reservoir (Suarez et al., 1997; Bergqvist et al., 2005). These findings suggest that PR might have a direct function on regulating the essential secretory activity in secretory cells in the isthmus and UTJ but PR does not influence the cellular dedifferentiation and regression in these segments which is in agreement with other reports that have suggested that there is a generally different grade of receptivity of the tissue and cell for ovarian steroid hormones such as the caudal parts of the oviduct (Abe et al., 1999; Hyde et al., 1989). Together, the observations indicate that regulation of relative expression of PR seems to differ in a phase- and tissue-specific way in female reproductive tissues.

In subepithelial connective tissue, the intensity and proportion of PR staining was detectable in all segments of the buffalo oviduct and higher during the follicular phase than the luteal phase of the estrous cycle. These results appear to confirm previous studies in rats, in which PR was found along the entire oviduct but with less intensity during diestrus (Pelletier et al., 2000). Valle et al. (2007) also found a negative correlation between PR and progesterone circulating concentrations, but not between PR and estrogen concentrations, indicating that progesterone inhibits the amount of PR in the connective tissue occurring in the surface epithelium. According to Mahmood et al. (1998), the essential role of PR in the connective tissue of the human oviduct is to regulate oviductal ciliary activity because the ciliated cells do not have PR staining. Kimmins and MacLaren (2001) suggest that PR in subepithelial connective tissue cells triggers the steroid responsiveness of the epithelium as shown from mice knock-out studies (Kurita et al., 2000). Furthermore, a lack of PR in ciliated epithelial cells suggests that there is no possibility of direct regulation of ciliogenesis of ciliated cells by estrogens or progesterone (Okada et al., 2003). Therefore, the plausible mechanism of ciliogenesis regulation by both steroid hormones could interact between epithelial and connective tissue cells via intermediate molecules produced by PR positive connective tissue cells which are clearly demonstrated in mouse female reproductive tract (Kurita et al., 2000).

Regarding the PRB staining intensity in the smooth muscle layer, a significant increase in the staining was

observed in the UTJ and isthmus during the follicular phase corresponding to that reported in heifers (Valle et al., 2007) and women (Amso et al., 1994). Intense nuclear staining of the muscular layer surrounding the oviduct provides some evidence for the importance of PR mediating motility. In addition, the different target regions of PR expression could possibly reflect function importance. Hunter et al. (1999) proposed progesterone interactions with sperm released from the sperm reservoir, i.e. UTJ and caudal isthmus. Since progesterone levels are not elevated directly in the oviduct around and after ovulation (Wijayagunawardane et al., 1998), minute levels of progesterone secreted by either preovulatory mature follicles or the early corpus luteum could unfold an effect via a countercurrent transfer to the oviduct. An upregulation of PR along the isthmus epithelium could indicate functional active hormone-receptor complexes which may lead to the controlled release of UTJ an isthmus bound sperm probably mediated through relaxation of surrounding oviductal muscular layer.

In conclusion, the present study demonstrates that PR expression was clearly detected in the Thai swamp buffalo oviduct and varied according to the segments of the oviduct as well as the marked changes of the periods of estrous cycle. Additionally, the PR was mediated by estradiol stimulation and progesterone inhibition to prepare the physiological functions for the events taking place in the buffalo oviducts.

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