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Original Article

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Abstract

Fas ligand (FasL) and its receptor (Fas) are tumor necrosis factor (TNF) members which are involved in the immune privileged organs, such as the cornea, testis and placenta, by triggering apoptosis in various cell types. The appearance of a Fas-FasL system might specify the immune privileged status of the buffalo oviduct where spermatozoa avoid eradication by female immune cells. The objective of this study was to scrutinize the FasL and Fas proteins by immunohistochemistry, FasL mRNA by RT-PCR and the apoptotic degrees by TUNEL analysis in the uterotubal junction (UTJ), isthmus, ampulla and infundibulum of swamp buffalo oviducts at follicular (n=5) and mid-luteal (n=5) phases. The Fas localization was scattered along the lining epithelium of all oviductal portions at both estrous cycle stages, whereas the intensity of FasL immunostaining was more noticeable in the epithelial cells of UTJ and isthmus at follicular phase and showed significant differences (p<0.05) compared to other segments and to the mid-luteal phase. FasL mRNA expression was detected in the epithelial cells of all oviductal segments and the intense of expression in UTJ and isthmus at follicular phase was greater than similar regions at mid-luteal phase and other segments. However, the TUNEL-apoptotic cells were rarely detected in all oviductal segments of swamp buffalo at both phases. The present results indicate that the appearance of Fas and FasL in the UTJ and isthmus which are the site of sperm reservoir, at follicular phase can be involved in Fas-FasL system that mediate the survival of spermatozoa and supports the immune privilege status in the swamp buffalo oviduct.

Keywords: estrous cycle, Fas-FasL, oviduct, sperm reservoir, swamp buffalo

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บทคัดย่อ

Fas Ligand ในท่อนำไข่กระบือปลักระยะฟอลลิคูลาร์และระยะลูเทียล

ไพศาล เทียนไทย [1] พงษ์พิสุทธิ์ จิวะเจริญ [1] เกรียงยศ สัจจเจริญพงษ์ [1] พรชลิต อัศวชีพ [2]*

Fas ligand (FasL) และ Fas ซึ่งเป็นตัวรับของ FasL อยู่ในกลุ่มของ tumor necrosis factor (TNF) โปรตีนทั้งสองชนิดมีส่วนเกี่ยวข้องกับการทำงานของอวัยวะที่เรียกว่า immune privileged organ เช่น กระจกตา อัณฑะ และรก โดยกระตุ้นให้เกิดภาวะการเสื่อมตายของเซลล์หลายชนิด การปรากฏของระบบ Fas-FasL อาจระบุถึงสภาวะ immune privilege ที่เกิดขึ้นภายในท่อนำไข่กระบือปลัก ซึ่งเป็นอวัยวะที่เซลล์อสุจิรอดพ้นจากการทำลายของระบบภูมิคุ้มกันในระบบสืบพันธุ์เพศเมีย วัตถุประสงค์ในการศึกษานี้ต้องการตรวจหาการปรากฏของโปรตีน Fas และ FasL ด้วยวิธีอิมมูนโนฮิสโตเคมี ตรวจหาการแสดงออกของ FasL mRNA ด้วยวิธี RT-PCR และตรวจระดับการเสื่อมตายของเซลล์ด้วยวิธี TUNEL ภายในท่อนำไข่กระบือปลักซึ่งประกอบด้วย รอยต่อปีกมดลูกกับท่อนำไข่ (Uterotubal junction, UTJ) อิสธ์มัส แอมพูลลา และอินฟันดิบูลัม ในระยะฟอลลิคูลาร์ (จำนวน 5 ตัว) และลูเทียลช่วงกลาง (จำนวน 5 ตัว) ผลการศึกษาพบการปรากฏของโปรตีน Fas กระจายอยู่ทั่วไปตามแนวเยื่อบุผิวของท่อนำไข่กระบือปลักทุกส่วนและทุกระยะของวงรอบการเป็นสัด ในทางตรงกันข้ามพบการปรากฏของโปรตีน FasL อย่างชัดเจนภายในเซลล์เยื่อบุผิวของรอยต่อปีกมดลูกกับท่อนำไข่และอิสธ์มัสในระยะฟอลลิคูลาร์อย่างมีนัยสำคัญทางสถิติ ($p<0.05$) เมื่อเปรียบเทียบกับท่อนำไข่ส่วนอื่นๆ หรือเปรียบเทียบกับระยะลูเทียลช่วงกลาง ผลการศึกษาด้วยวิธี RT-PCR พบการแสดงออกของ FasL mRNA ในเยื่อบุของท่อนำไข่ทุกส่วน แต่ระดับความเข้มในการแสดงออกในส่วนรอยต่อปีกมดลูกกับท่อนำไข่และอิสธ์มัสระยะฟอลลิคูลาร์มีความเข้มกว่าเมื่อเปรียบเทียบกับท่อนำไข่ส่วนเดียวกันระยะลูเทียลช่วงกลาง และท่อนำไข่ส่วนอื่นๆ อย่างไรก็ดี ผลการตรวจสอบการเสื่อมตายของเซลล์โดยใช้วิธี TUNEL พบได้เพียงเล็กน้อยในท่อนำไข่ทุกส่วนของกระบือปลักทั้งสองระยะของวงรอบการเป็นสัด ผลการศึกษาดังกล่าวระบุว่าการแสดงออกของ Fas และ FasL ในบริเวณรอยต่อปีกมดลูกกับท่อนำไข่และอิสธ์มัสซึ่งเป็นบริเวณกักเก็บเซลล์อสุจิในระยะฟอลลิคูลาร์ เกี่ยวข้องกับการทำหน้าที่ของระบบ Fas-FasL ที่ส่งผลให้มีการรอดชีวิตของเซลล์อสุจิ และสนับสนุนสภาพของ immune privilege ที่อาจเกิดขึ้นในท่อนำไข่กระบือปลัก

คำสำคัญ: วงรอบการเป็นสัด Fas-FasL ท่อนำไข่ บริเวณกักเก็บเซลล์อสุจิ กระบือปลัก

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Introduction

Sperm storage and capacitation, ovum pick-up, fertilization including early embryonic growth are critical situations taking place in the cattle oviduct (Hunter and Wilmut, 1984; Lefebvre et al., 1995). The mucosal surface in the female reproductive tract is protected by innate and adaptive immune defense regulations (Wira et al., 2005). The innate defense mechanism is composed of the tight junction of epithelial cells, antimicrobial secretions and phagocytic immune cells, whereas the intraepithelial lymphocytes primarily serve as the adaptive mechanism (Mowat, 2003). After insemination, large amounts of spermatozoa that contain foreign proteins are demolished by neutrophil phagocytosis and other immune cells responses within the uterine horns (Katila, 2001; Kaeoket et al., 2003; Matthijs et al., 2003). After rapid transport in cattle, hundreds of spermatozoa are arrested in the uterotubal junction (UTJ) and adjoining isthmus, the site defined as the sperm reservoir, where they can survive the micro-environment for several hours until release when the ovulation occurs (Hunter and Wilmut, 1984). Unlike the uterine horns, these spermatozoa are able to hide away the mucosal immune response of the oviduct. In inseminated pigs at pre-ovulation, Rodriguez-Martinez et al. (1990) indicated that the neutrophils were absent from the UTJ and the caudal isthmus was irrelevant to the appearance of boar spermatozoa. Furthermore, the intraepithelial immune cell numbers in non-inseminated swamp buffalo oviduct were very low at follicular phase in UTJ compared to the other oviductal segments (Tienthai et al., 2008). According to these observations, some regions of the buffalo oviduct, particularly in the UTJ and adjacent isthmus, might function as an immune privileged organ corresponding to the testis (Lee et al., 1997; Koji et al., 2001), the placenta (Hammer et al., 1999; Kauma et al.,

1999), or the cornea in the eyes (Stuart et al., 1997) where cells expressing foreign proteins escape rejection.

Immune privileged organs and tissues generally have a specific regulation to allow the foreign proteins to survive the immune responses and the Fas-Fas ligand (FasL) system has been implicated as an essential mediator for this regulation (Griffith et al., 1995). FasL is a type II transmembrane protein belonging to the tumor necrosis factor (TNF) superfamily and the Fas receptor is a type I transmembrane protein with a death domain in its cytoplasmic region that stimulates an apoptotic signal when binding to FasL (Suda and Nagata, 1994). It is believed that the localization or expression of FasL accords to the immune privilege by inducing Fas-mediated apoptosis in lymphocytes that distribute into FasL-bearing tissue (Nagata, 1997). For instance, the remarkable appearance of FasL in the glandular epithelium and the decidual cells in the placenta and the capability of trophoblasts to induce FasL-dependent apoptosis, indicate the role of Fas-FasL system in maternal immune tolerance toward the fetus (Mor et al., 1998; Kauma et al., 1999). The detection of Fas-FasL interaction in the swamp buffalo oviduct can explain the local immune tolerance that prevents the spermatozoa from cytotoxic T lymphocytes. Therefore, the present study was performed to investigate Fas-FasL immuno-localization, FasL mRNA expression and apoptotic analysis in all segments of the swamp buffalo oviduct during the follicular and mid-luteal phases.

Materials and Methods

Animals and tissue collection: Female swamp buffalo (n=20) of various ages (2-8 years) were slaughtered at a local abattoir and their genital tracts were immediately collected and reserved in a cool container (~4^{o}C) for at least 30-45 min until being processed in the laboratory. The reproductive organs were observed and the ovarian characteristics were determined by the morphological appearance of the corpus luteum (Ali et al., 2003), i.e. follicular (n=10) and mid-luteal (n=10) phases. The swamp buffalo oviducts were divided into uterotubal junction (UTJ), isthmus, ampulla and infundibulum. The samples for RNA extraction (the follicular phase, n=5 and the luteal phase, n=5) were promptly deep-frozen in liquid nitrogen (LN_2), whereas the specimens for immunohistochemistry and TUNEL assay (follicular phase, n=5 and luteal phase, n=5) were immersed in 4% paraformaldehyde at 4^{o}C until being embedded in paraffin. Tissue blocks were cut at 4 µm thickness and serial sections were mounted on Superfrost plus slides (Menzel-Graser, Frieburg, Germany) for Fas, FasL immunohistochemical and TUNEL techniques.

I***mmunohistochemical procedure for Fas and FasL***: After deparaffinization and rehydration, tissue sections were quenched with 3% hydrogen peroxide (H_2O_2) in methanol and washed in phosphate buffer saline (PBS, pH 7.4). For the primary antibody, mouse anti-human Fas monoclonal antibody (B-10, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit anti-rat FasL polyclonal antibody (N-20, Santa Cruz) were used. Briefly, sections were blocked with 10% normal horse serum (for FasL) or normal goat serum (for Fas), prior to incubation overnight at 4^{o}C in a humidity chamber with a 1:100 dilution of Fas antibody or a 1:150 dilution of FasL antibody. After rinsing with PBS, bound antibody was detected by the avidin-biotin-peroxidase method, using reagents of the Vectastain ABC kit (Vectastain ABC-Elite, Vector Laboratories, Burlingame, CA, USA). Biotinylated horse anti-mouse IgG (Vector Laboratories) at a dilution of 1:500 was used for Fas, whereas biotinylated goat anti-rabbit IgG (Vector Laboratories) at a dilution of 1:400 was used for FasL. For both Fas and FasL antigens, bound peroxidase were reacted with 3,3′-diaminobenzidine substrate (DAB kit, Vector Laboratories) with H_2O_2 to give a brown reaction product. All slides were counterstained with hematoxylin and mounted in glycerin-gelatin. As negative controls, adjacent sections were reacted in parallel with a substitution of mouse IgG (dilution 1:100, DAKO, Glostrup, Denmark) for primary antibodies. Ovarian sections prepared from adult mice were used as positive controls. The stained slides were then investigated under light microscope (BX50, Olympus, Tokyo, Japan) equipped with a digital camera Micropublisher 5.0 (Qimage, Surrey, BC, Canada) and software program (Image-Pro Plus 6.0 (Media Cybernatics Inc., Bethesda, MD, USA). Examination of Fas and FasL positive immunostaining in the surface epithelium of all oviductal segments was carried out by blind preparation. The intensity of positive staining was categorized into four different scores as follows: no staining, 0; weak, 1; moderate, 2 and strong, 3.

Extraction of total RNA and reverse transcription-polymerase chain reaction (RT-PCR): The tubal epithelium from the UTJ, isthmus, ampulla and infundibulum of swamp buffalo oviduct was scraped using the blunt side of a scalpel blade. Total RNA was isolated from the epithelial cells by use of the RNeasy mini kit (QIAGEN GmbH, Hilden, Germany). Synthesis of cDNA and PCR was performed using Transcriptor One-Step RT-PCR kit (Roche, Mannheim, Germany). Amplification conditions were as follows: denaturation 94^{o}C for 30 sec, annealing 55^{o}C for 30 sec, extension 72^{o}C for 30 sec, for 44 cycles. The primers used were designed for bovine FasL (Sigma-Genosys Ltd., Pampisford Cambridgeshire, UK). Sense and anti-sense specific primers were as follows: 5′-TATTCCAAAGTATACTTCCGGGGT CA-3′ and anti-sense 5′-ACTGCC CCCAGGTAGCTGCTG-3′ (Genebank accession number U95844).

Apoptotic analysis by TUNEL assay: After being deparaffinized and rehydrated, the tissue sections were pretreated with proteinase K for 15 min at room temperature (RT). The Terminal Deoxynucleotidyl Transferase-mediated dUTP nick-end labeling (TUNEL) assay to evaluate apoptotic cells using an ApopTag® Peroxidase *In Situ* Apoptosis Detection Kit (Chemicon International Inc., CA. USA) was performed according to the manufacturer's

instructions. In brief, endogenous peroxidase activity was reduced with 3% H_2O_2 in PBS for 5 min at RT. After rinsing, the slides were treated with equilibration buffer for 5 min at RT and then incubated with TdT enzyme for 1 hr at 37°C using a parafilm cover. A stop buffer was added for 10 min at RT, followed by washing in PBS and incubating with an anti-digoxigenin conjugate for 30 min at RT. To visualize the labeled 3′-OH ends of DNA fragments, 3,3′-diaminobenzidine substrate (DAB kit, Vector laboratories) with H_2O_2 was applied for 1-3 min. The slides were then rinsed in distilled water, counterstained with Mayer's hematoxylin and mounted with glycerine gelatin. Negative control sections were incubated with PBS instead of TdT working enzyme whereas canine lymph nodes with lymphoma were used as a positive control. An ocular micrometer with 25 squares corresponding to 15,625 μm^2 at magnification 400x was used for counting the TUNEL-positive cells along the lining epithelium. For each oviductal segment, 20 microscopic areas were randomly selected for evaluation.

Statistical analyses: Data was handled and analyzed using the SAS statistical package (version 9.0, SAS Institute Inc., Cary, NC, USA). The intensity score of Fas-FasL immunostaining and the number of TUNEL-positive cells were compared between the oviductal segments and estrous cycle stages using one-way factorial ANOVA. Differences between means were determined by a student t-test, p values <0.05 were considered statistically significant.

Results

Immunolocalization of Fas proteins: In control sections, Fas-positive immunolocalization appeared in the cytoplasm of follicular granulosa cells surrounding the oocyte in the mouse ovary (Fig. 1a),

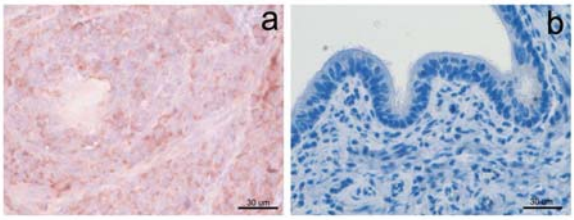

Figure 1 Control sections for Fas immunohistochemistry. Mouse ovarian section was used as the positive control (a); Omission of the primary antibody and substitution by a mouse IgG on UTJ section was used as the negative control (b).

immunolabeling was present in the cytoplasm of epithelial cells, especially in the apical part of cells, and the staining was dispersed along the lining epithelium at both phases of the estrous cycle (Fig. 2a-h). The moderate to strong staining of Fas-positive labeling was varied along the oviduct, but no obvious differences in the intensity scoring could be observed among portions (Fig. 3A) or between phases of the estrous cycle (Fig. 3B).

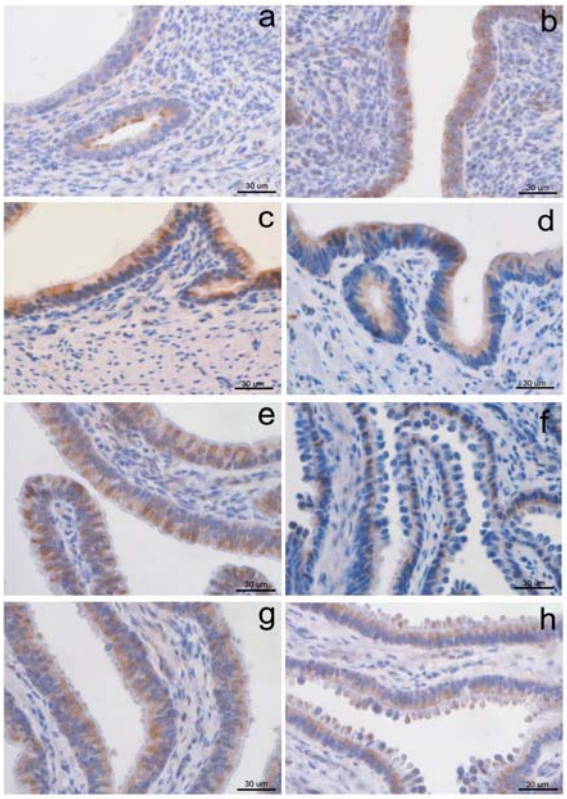

Figure 2 Fas immunolocalization in the epithelium of the UTJ (a, b), isthmus (c, d), ampulla (e, f), and infundibulum (g, h) of the swamp buffalo oviducts at the follicular (a, c, e, g) and mid-luteal (b, d, f, h) phases of the estrous cycle.

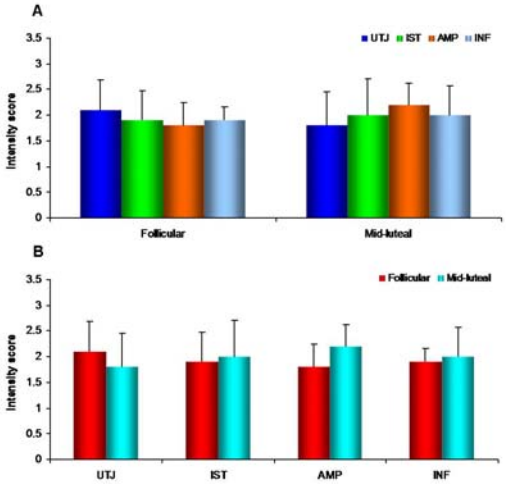

Figure 3 Intensity scores of Fas immunostaining in the lining epithelium of the UTJ, isthmus, ampulla and infundibulum of the swamp buffalo oviduct during the follicular and mid-luteal phases of the estrous cycle. (A) Staining intensity was compared between segments at the same phase and (B) was compared between phases. Values are depicted as mean±SEM with different labels (a, b) being significantly different (p<0.05).

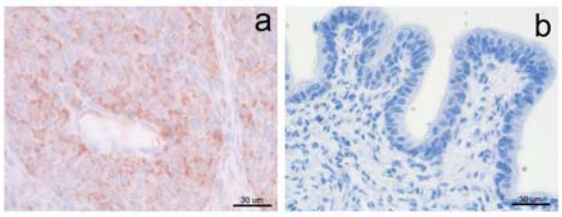

Figure 4 Control sections for FasL immunohisto chemistry. Mouse ovarian section was used as positive control (a); Omission of the primary antibody and replacement with a mouse IgG on UTJ section was used as the negative control (b).

Immunolocalization of FasL proteins: In the control sections, FasL-positive immunolocalization was also clearly found in the cytoplasm of follicular granulosa cells (Fig. 4a), whereas there was no immunostaining on the negative controls (Fig. 4b). Similarly to the Fas proteins, FasL-positive staining was shown in the cytoplasm of epithelial cells, which could not identify the cell types, along the oviductal segments (Fig. 5). However, the intense immunolocalization of FasL protein was conspicuously present in the epithelial cells of the swamp buffalo oviduct, especially in the UTJ and isthmus, at the follicular phase (Fig. 5a, c, e, g) compared to the mid-luteal phase (Fig. 5b, d, f, h). At the follicular phase, manual intensity scoring confirmed that the FasL labeling in the UTJ and isthmus was significantly higher (p<0.05) than the ampulla and infundibulum (Fig. 6A). In addition, the FasL intensity in the UTJ and isthmus at the follicular phase was significantly greater (p<0.05) than the same regions at the luteal phase (Fig. 6B).

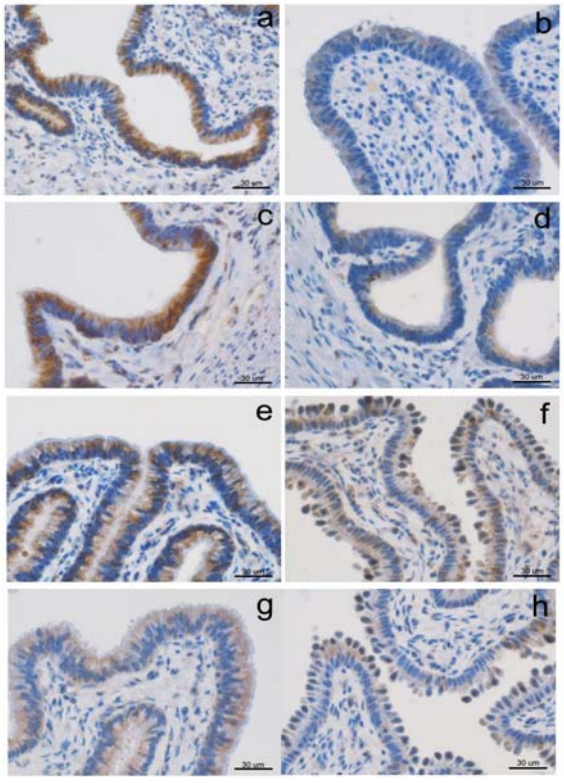

Figure 5 FasL immunolocalization in the epithelium of the UTJ (a, b), isthmus (c, d), ampulla (e, f), and infundibulum (g, h) of the swamp buffalo oviducts at the follicular (a, c, e, g) and mid-luteal (b, d, f, h) phases of the estrous cycle.

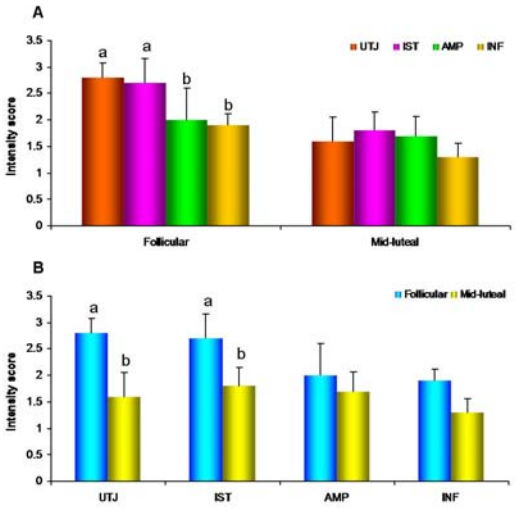

Figure 6 Intensity scores of FasL immunostaining in the lining epithelium of the UTJ, isthmus, ampulla and infundibulum of the swamp buffalo oviduct during the follicular and mid-luteal phases of the estrous cycle. (A) Staining intensity was compared between segments at the same phase and (B) was compared between phases. Values are depicted as mean±SEM with different labels (a, b) being significantly different (p≤0.05).

FasL mRNA expression: An expected 168 bp band was investigated in all samples of the swamp buffalo oviduct at both estrous cycle stages (Fig. 7). At follicular phase, the FasL mRNA expression was more abundant in the UTJ and isthmus than in the ampulla and infundibulum as well as the oviductal segments at luteal phase.

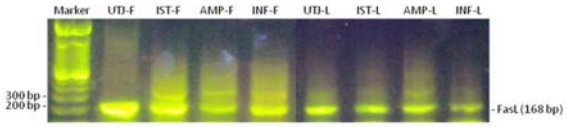

Figure 7 FasL mRNA expressions in the epithelium of the uterotubal junction (UTJ), isthmus (UST), ampulla (AMP) and infundibulum (INF) of the swamp buffalo oviduct during the follicular (F) and mid-luteal (L) phases of the estrous cycle.

Table 1 Number of TUNEL-positive apoptotic cells (mean±SD/ area of ocular micrometer 15625 μm²) in different segments of swamp buffalo oviduct at follicular and mid-luteal phases

Oviductal segments/ Estrous cycle	UTJ	Isthmus	Ampulla	Infundibulum
Follicular	0.24±0.52	0.08±0.28	0.16±0.37	0.12±0.33
Mid-luteal	0.12±0.33	0.04±0.20	0.20±0.50	0.12±0.33

Detection of apoptosis: The lymph nodes (lymphoma) which were used as positive controls for TUNEL assay demonstrated numerous positive apoptotic cells or apoptotic bodies (brown staining) throughout the tissue sections (Fig. 8a), whereas none of the cells in the negative control (ampulla section) were labeled (Fig. 8b). In the swamp buffalo oviduct, however, very few apoptotic cells were detected in any segments at both phases of the estrous cycle (Fig. 9) and the number of TUNEL-positive cells was not significantly different (p>0.05) between segments or estrous phases (Table 1).

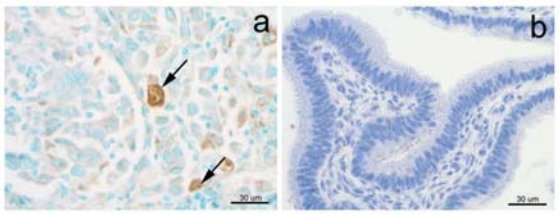

Figure 8 Control sections for TUNEL assay. Canine lymph node (lymphoma) section (a) showed TUNEL-positive brown staining (arrows); UTJ section replacement with mouse IgG was used as the negative control (b).

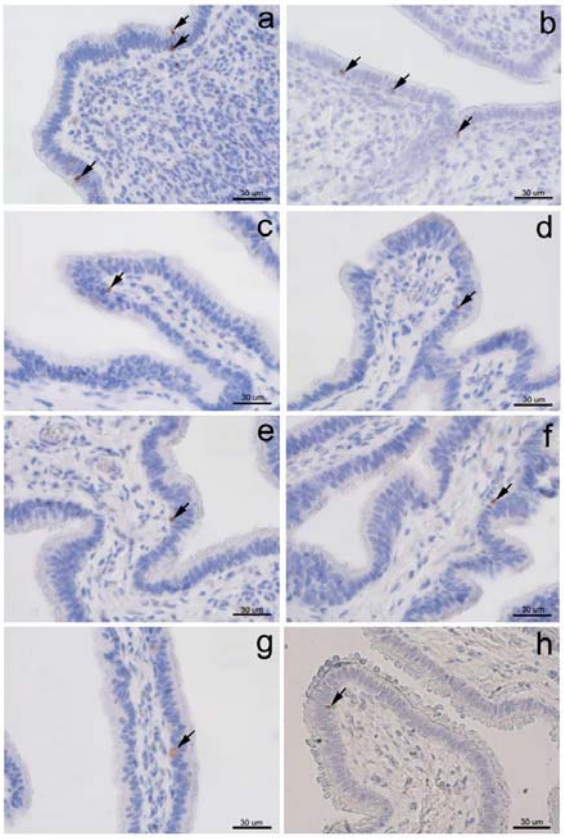

Figure 9 Detection of apoptotic cells (arrows) by TUNEL assay in the epithelium of the UTJ (a, b), isthmus (c, d), ampulla (e, f), and infundibulum (g, h) of the swamp buffalo oviducts at the follicular (a, c, e, g) and mid-luteal (b, d, f, h) phases of estrous cycle.

Discussion

The present study confirmed that protein localization and mRNA expression of FasL was found in the swamp buffalo oviductal epithelium, especially in the UTJ and adjacent isthmus (the site of sperm reservoir), at the follicular phase which was the time of sperm storage and fertilization. Contrastly, Fas immunostaining was clearly present along the lining epithelium of all oviductal portions at all estrous cycle stages. Although the apoptotic cells were rarely observed by TUNEL assay, the present investigation suggested that a regulation of Fas-FasL system occurred within the sperm reservoir of the swamp buffalo oviduct.

It is known that the main functions of the cattle oviduct in relation to critical events, i.e. sperm transport, oocyte collection, fertilization and initial embryonic growth, take place in 2-3 days subsequent to the beginning of the estrus period (Lefebvre et al., 1995; Suarez, 2002). Several studies found that spermatozoa contained proteins that were considered to be a foreign to the female immune system and the presence of spermatozoa and seminal plasma in the uterine horns after mating or artificial insemination induced the distribution of polymorphonuclear leukocytes and other immune cells from the subepithelial connective tissue layer of the endometrium to eliminate them (Katila, 2001; Kaeoket et al., 2003; Matthijs et al., 2003). Hunter et al. (1991) found that the caudal isthmus and UTJ functioned as the sperm reservoir in cattle where the spermatozoa remained viable before fertilization. Altogether, this specific region, e.g. in pigs, demonstrates a minimal immune response by leukocytes after insemination (Rodriguez-Martinez et al., 1990) corresponding to the swamp buffalo oviduct that shows a small number of immune cells within UTJ and isthmus epithelium throughout the estrous cycle (Tienthai et al., 2008). One of the essential factors involved in sperm survival in the sperm reservoir is the presence during the pre-ovulatory stage of mucous hyaluronan that contains the immunologically static condition to assist the immersed spermatozoa escape identification by immune cells (Tienthai et al., 2000; Bergqvist et al., 2005[a]). However, this event does not explain the decrease of the immune cells in the sperm reservoir of various species and recently the presence of Fas-FasL system can be detected in the bovine oviduct (Bergqvist et al., 2005[b]) which is thought to have a major role in the limiting of the immune cell numbers in the cattle oviduct.

We know that immunologically privileged sites are the regions of the body where the immune system does not appear to function (Griffith, 1995) and the Fas-FasL system apoptotic pathway has been demonstrated to play an important role in the immune system by stimulating the activation inducted suicide of T-lymphocytes and other immune cells (Steller, 1995). In the present study, the FasL immunostaining was found along the epithelial lining of the swamp buffalo oviduct corresponding to the bovine oviduct (Bergqvist et al., 2005[b]), however, our result confirmed that a strong intensity of FasL

protein was only present in the epithelial lining of the UTJ and adjacent isthmus at the pre-ovulatory phase in relation to the expression of FasL mRNA. Therefore, the site of the sperm reservoir (UTJ and caudal isthmus) in the swamp buffalo oviduct might also acquire this special mechanism to selectively eliminate immune cells and temporarily maintain sperm ability and also the early embryo. Nagata and Golstein (1995) reported that activated T lymphocytes that expressed Fas and FasL were sensitive to Fas-induced apoptosis indicating that the activated T lymphocytes commited suicide or even killed each other. Recently, the Fas-FasL system was also associated with the process in the decline of natural killer (NK) cells by apoptosis (Kusakabe et al., 2005) and the apoptotic signals produced by trophoblastic FasL regulate the immune traffic including maintaining maternal tolerance by preventing the activated T lymphocytes from entering the fetal cellular compartment (Xerri et al., 1997). This data confirms the expressions of Fas and FasL in both activated T cells and NK cells, and there is the possibility that apoptosis induction in the sperm reservoir of swamp buffalo oviduct will occur when these immune cells (Fas-bearing cells) are distributed into the epithelial lining which demonstrates the intense FasL immunostaining. In the present study, Fas-FasL localization in the cell surface of the intraepithelial or subepithelial immune cells in swamp buffalo oviducts was not observed, therefore, cryostat tissue sections instead of paraffin sections would be required to investigate Fas-FasL immunolocalization (Kusakabe et al., 2005) in any further study.

In the present study, the localization of FasL protein was shown in the apical part of the oviductal epithelial cells corresponding to earlier studies in mouse (Imarai et al., 2005) and cow (Bergqvist et al., 2005[b]) oviducts. Importantly, our technique with paraffin sections was unable to identify which types of oviductal epithelial cells reacted to FasL immunostaining. However, the previous studies by immune-electron microscopic analysis in melanoma cells (Adreola et al., 2002) and ocular epithelial cells (McKechnie et al., 2006) reported that FasL proteins were localized as multi-vesicular patterns, therefore, FasL proteins could be bound to the secretory vesicles or granules. These observations assumed that the positive staining of FasL at the apical portions was in the location of secretory granules within the secretory cells of epithelial cells since being carefully noticed in positive FasL immunolabeling in the UTJ (Fig. 5a) and isthmus (Fig. 5c) of swamp buffalo oviducts. Considering the estrous cycle stage, both FasL protein and mRNA in this study obviously appeared during the follicular phase compared to the mid-luteal phase and one possible reason is that the presence of FasL can be regulated by the influence of estrogen. Song et al. (2002) suggested that FasL proteins expression increased in Ishikawa cells and primary cultures of uterine glandular cells treated with estrogen. Furthermore, Sapi et al. (2002) indicated that high levels of estrogen during estrus definitely upregulated the expression of FasL proteins and mRNA intensity in ovarian epithelial cells and ovarian tissues. These observations not only suggest that the abundance of Fas protein localization and mRNA expression are influenced by estrogen during the follicular phase, but also confirms that FasL is biologically active in the swamp buffalo oviduct.

One question raised in our study was whether the Fas-FasL system in the swamp buffalo oviduct might trigger apoptosis in the epithelial cells during the estrous cycle as the presence of Fas-FasL was found in the epithelium of all oviductal portions. In cattle oviducts, the epithelial extrusion of secretory cells markedly occurred in the ampulla and infundibulum at the luteal phase (Abe and Oikawa, 1993; Tienthai et al., 2008; 2009) and this phenomenon was found in the present study (Fig. 2f, h and Fig. 5f, h) as well. Several investigators have reported that the cytoplasmic and nuclear extrusions of oviductal epithelium were influenced by high levels of progesterone and were related to apoptotic events (Verhage et al., 1984; 1990). However, Steffl et al. (2008) recently concluded that this epithelial protrusion was a characteristic feature of non-apoptotic cell loss of secretory (non-ciliated) cells in large animals and dogs which were a species with long luteal phase. In our study, a fewer TUNEL-apoptotic cells were demonstrated in all segments of the swamp buffalo oviduct at both estrous stages similar to the bovine oviduct (Bergqvist et al., 2005[b]) suggesting that there was no correspondent between the Fas-FasL localization and apoptosis in the swamp buffalo oviductal epithelium. However, we know that the data associated with apoptosis in large domestic animals' oviducts is very rare and those studies performed only TUNEL assay which can detect late and short stages of apoptosis (Grossmann et al., 1988), while the Fas-FasL system may be expressed at an earlier stage. To prove exact apoptotic events, the specific and sensitive markers of apoptosis which detect cells in early stages, such as the immunohistochemical detection of apoptotic cells using an anti-single-stranded DNA (anti-ssDNA) antibody (Kawarada et al., 1998; Steffl et al., 2008), might be investigated in the cattle oviduct. Based on our results, however, the FasL immunolocalization and mRNA expression by the epithelial cells of sperm reservoir in the swamp buffalo oviduct might act as an immune privileged site and could induce apoptosis in Fas-bearing immune cells by the mechanism of direct cell-cell contact.

In conclusion, the present study indicates the appearance of Fas-FasL proteins and FasL mRNA in the epithelial cells of the swamp buffalo oviduct. Importantly, FasL was particularly present in the UTJ and isthmus at the follicular phase supporting the theory that sperm reservoir in the swamp buffalo oviduct serves as an immune privileged site. However, the mechanisms of the Fas-FasL system in relation to the presence of FasL in various types of immune cells and the apoptotic events by other techniques in the swamp buffalo oviduct still require further investigation.

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