



FULL PAPER

Theriogenology

Characterization of inflammatory responses by cervical cytology, cytokine expression and ultrastructure changes in a goat subclinical endometritis model

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ABSTRACT. Endometritis is a common puerperal disease in livestock. Thus, a practical clinical test for the establishment of diagnosis is needed. We developed a goat model for subclinical endometritis diagnosis by examining cytological, ultrastructural features and molecular expression in cervical discharge collected by cervical cytobrush method. A suspension of *E. coli* was infused into the uterine horn of goats, and cervical discharge, peripheral blood and endometrial biopsy samples were collected before inoculation and at 3, 6, 12, 24, 72, 120 and 168 hr post-inoculation (pi). In experimental goats, total leukocytes increased at 6–12 hr pi in hematological examinations, abundant neutrophils were observed in cervical discharge smears, and some micro-villi shortened or were lost from the epithelium at 3 hr pi, with some inflammatory cells infiltrated into the uterine glands and lamina propria. The mRNA expression levels of the Toll-like receptor 4 (TLR4), cytokines and β -defensin-2 increased significantly within 12 hr pi and returned to pre-inoculation levels at 7 day pi. The results showed that subclinical endometritis was simulated in this bacterial infusion goat model, and the evaluation of cervical cytology was in good agreement with molecular changes of cytokines in cervical discharge, hematology and histology. We thus conclude that the simple cervical cytobrush technique can effectively obtain cervical discharge samples for cytology examination in the early diagnosis of subclinical endometritis in goats.

KEY WORDS: cervical cytology, goat, inflammatory cytokine, subclinical endometritis, ultrastructure

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Endometritis caused by pathogenic organisms is common in peripartum livestock, leading to delayed uterine involution, reduced milk yield, fertilization failure, infertility and even culling [15, 27]. The incidence of uterine infections is 10% to 17% in dairy cows [20] and 1.53% to 3.21% in goats [23], causing major economic losses in the animal breeding industry. The development of endometritis depends on species, the number of bacteria colonizing the endometrium and the host immune response [20]. Bacterial organisms are considered to be the predominant cause of endometritis, with *Escherichia coli* (*E. coli*), *Arcanobacterium pyogenes* and *Staphylococcus aureus* accounting for the most common isolates [21, 26]. After microbial challenge, the innate immune system components including Toll-like receptors (TLRs), acute phase proteins (APPs), antimicrobial peptides (AMPs), and polymorphonuclear leukocytes (PMNs) or neutrophils are rapidly activated to clear the bacteria or related inflammatory products [8]. PMNs migrate from peripheral circulation into the uterine cavity and perform phagocytosis and meanwhile synthesize cytokines and chemokines, including IL-1, IL-6, IL-8, IL-18 and TNF- α , which can subsequently recruit more neutrophils and modulate their phagocytic functions [28]. Endometritis is divided into clinical and subclinical endometritis. The clinical endometritis is characterized by the presence of purulent or mucopurulent uterine exude in the vagina and fetid red-brown watery uterine discharge, while the subclinical endometritis has unapparent infection symptoms and is thus somehow difficult to be diagnosed due to a lack of noticeable clinical symptoms [15].

An accurate, prompt diagnosis of subclinical endometritis using a practical method is critical in veterinary practice. A few

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techniques are applied in the diagnosis of endometritis in cattle and mare, including hematology, bacteriology, vaginoscopy, ultrasonography, transrectal palpation, cytology and histological examination [22], while in commercial sheep and goat flocks, diagnosis of endometritis is usually done by inspection of the pelvic region and vaginal examination using vaginal scope [12]. Several studies reported that cervical cytology was used for the diagnosis of endometritis in cows due to advantages, such as easy procedure, short time reporting and simple equipment [14, 29], while other diagnosis methods including vaginoscopy, ultrasonography, cytokines detection and histopathology were characterized by a long results waiting time and the need of special tools. In addition, some researchers examined the transcripts or serum levels of inflammatory cytokines, and APPs reflect the systemic immune status as diagnostic indicators of subclinical endometritis [11].

Although at present, the uterine cytology is the most prevalent method for subclinical endometritis diagnosis in animals [7], a simpler and more practical method is still needed. Our previous work showed that endometrial cytological changes were consistent with the endometrial histopathology changes [19], and it was also noticed that in cow subclinical endometritis, uterine and cervical cytology showed similar features, which made us hypothesize that the more practical operation of cervical cytology may replace histology and uterine cytology for the early diagnosis of subclinical endometritis.

To test this hypothesis, we used *E. coli* to infuse the uterine of goat to establish a subclinical endometritis model and characterized cervical cytological, histopathological and molecular biological features in cervical discharge collected by cytobrush method. We found that the cervical cytological examination is a novel, effective and practical method for the early diagnosis of subclinical endometritis in goats.

MATERIALS AND METHODS

All experimental procedures were approved by the Animal Care and Use Committee of Yangzhou University (approval ID: SYXK (Su) 2007–0005). All animal experimentation described was conducted in accord with accepted standards of animal care.

Animals, surgery and experimental design

We used 12 postpartum dairy goats, 2–4 years old, 40–50 kg and appearing clinically healthy. Cervical cytological examinations were normal, and neither lochia nor abnormal cervical discharge was observed up to 25 days after kidding and prior to the selection for this study. Goats were individually housed in pens and fed a commercial hay and grain mixture, with *ad libitum* access to tap water. Uterine catheters were used for the infusion of *E. coli* or PBS suspension into the uterine horns. A detailed procedure for uterine catheterization and the experimental design can be found in the primary paper on this investigation [19].

Blood collection and hematology analysis

Blood samples (5 mL) were taken from the jugular vein and collected with EDTA anticoagulant tube (Becton, Dickinson & Co., Franklin Lakes, NJ, U.S.A.) at 0, 3, 6, 12, 24, 72, 120 and 168 hr post-inoculation (pi), and used for a white blood cell and leukocyte differential counts using a hemocytometer (BC3000, Mairui Ltd., Shenzhen, China).

Cervical discharge collection and cervical cytological examination

Cervical discharge samples used for cytological examination were collected with a sterile cytobrush (Wallcath Surgical Devices Corporation, Trumbull, CT, U.S.A.). The cytobrush was modified with the brush placed in a sanitary plastic tube (15 cm in length and 5 mm in diameter) prior to transvaginal insertion. For sample collection, goats were restrained in the standing position, and the perineal area was cleaned with sterile gauze sponges (Medline Industries, Inc., Mundelein, IL, U.S.A.). A sterilized sanitary plastic tube containing the cytobrush was inserted into the vagina until it reached the external cervical os. Cervical discharge samples were collected by rotating the cytobrush in a clockwise direction, and then, the cytobrush was retracted into the plastic tube prior to removal from the uterus. Before and after sampling, the cytobrush was weighed and marked as W1 or W2, and the weight of the cervical discharge was W2–W1. Next, the cytobrush was rolled onto a glass slide to allow air dry, and then, the modified Wright–Giemsa stain was performed. The %PMNs was calculated as previously described [19]. Briefly, a minimum of 100 cells were observed at $\times 400$ and $\times 1,000$ by a microscope (Eclipse80i, Nikon Corp., Tokyo, Japan) to determine the percentage of PMNs. Finally, the remaining discharge on the cytobrush was collected into a tube with RNase-free water for RNA extraction.

Endometrial biopsy and ultra-micro structure examination

Endometrial biopsy samples were collected at the time of uterine infusion (0 hr) and at 3, 6, 12, 24, 72, 120 and 168 hr pi. While the goats were restrained in a standing position, epidural anesthesia was performed (3 mL of 2% lidocaine), the perineum was cleaned, and endometrial biopsies were obtained via endoscopy. For this procedure, a vaginal dilator was used to distend the vagina, and the endoscope was guided into the vagina and through the cervical lumen until it reached one of the uterine horns. The biopsy instrument was introduced through the endoscope, and when the biopsy jaws were visualized, they were opened and gently pressed against the endometrial mucosa before they were closed to obtain a sample. Samples were collected by alternating between the right and left uterine horns on successive goats. All samples were fixed in 2.5% glutaraldehyde (Sigma-Aldrich, St. Louis, Mo, U.S.A.) for ultra-micro structure examination. Standard microscope techniques procedures were followed as previously described by Timmerman *et al.* [24]. Briefly, for scanning electron microscopy (SEM), specimens were washed with PBS, dehydrated in a graded ethanol series, treated with isoamyl acetate for substitution, dried by critical point drying method, coated in gold and examined using an electron microscope (S-4800II, Hitachi High-Technologies Corp., Tokyo, Japan). For transmission electron microscopy (TEM), tissues were washed with PBS, post-fixed in cold 1% osmium tetroxide, dehydrated in an ethanol series,

infiltrated and embedded in epoxy resins. Ultrathin sections were obtained with an ultra-microtome equipped (EM UC6, Leica Microsystems Inc., Wetzlar, Germany) with glass knives, stained with 0.5% ethanolic uranyl acetate and lead citrate, and examined using a transmission electron microscope (Tecnai 12, Philips Co., Eindhoven, Netherlands).

RNA extraction and real-time quantitative reverse-transcription (RT) PCR

Total RNA was extracted from cervical discharge samples using Trizol (Life Technologies, Carlsbad, CA, U.S.A.) and phenol-chloroform methods. A portion of the total RNA was treated with DNase (Life Technologies) to exclude genomic DNA contamination. The quantity and quality of the extracted RNA were assessed using a Nanodrop spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, U.S.A.) and the Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA, U.S.A.), respectively. Total RNA was eluted in 50 μ l (the RNA yield ranged from 400 to 1,000 μ g/ml), and the absorption ratio ($OD_{260\text{nm}}/OD_{280\text{nm}}$) was between 1.8 and 2.2.

The mRNA levels of target genes in cervical discharge were assessed by quantitative real-time RT-PCR. Total RNA (1 μ g) from each sample was reverse transcribed (10 μ l) according to the manufacturer's instructions (Life Technologies). Each real-time PCR reaction was performed using 1.2 μ l cDNA, 0.5 μ l primer (forward and reverse primers, 10 μ M) and 0.5 μ l Platinum SYBR Green qPCR Super Mix (Life Technologies) in a final volume of 20 μ l per reaction. Primers were designed to span an intron within the corresponding genomic sequence using Primer5 software (Premier Biosoft, Palo Alto, CA, U.S.A.) and synthesized by Invitrogen. Primer sequences can be found in our previous paper [19]. Cycling parameters for real-time PCR reactions were: 95°C for 2 min, 40 cycles of 95°C for 10 sec, 60°C for 30 sec and 72°C for 30 sec, using a 7500 Real-Time PCR System (Applied Biosystems, Life Technologies). The quantitative RT-PCR reactions were performed in triplicate for each sample, and the mean value was used to calculate mRNA expression levels. Six biological replicates were measured for each group.

The fold change (n-fold) for the expression of each gene was calculated using the relative quantification method ($2^{-\Delta\Delta Ct}$), using β -actin as an endogenous control. The average ΔCt for samples collected at 0 hr was used as the calibrator for each sample. Briefly, using *TLR4* as an example, given that ΔCt normalized target = *TLR4* Ct – β -actin Ct and $\Delta\Delta Ct$ = ΔCt normalized target – ΔCt normalized calibrator, the n-fold ($2^{-\Delta\Delta Ct}$) were calculated.

Statistical analyses

The effect of intrauterine infusion of *E. coli* on repeated measures of molecular mRNA expression levels and plasma protein concentrations were analyzed by ANOVA, using the MIXED procedure of SAS 9.1 (SAS Institute Inc., Cary, NC, U.S.A.). A first-order autoregressive covariance structure was defined to account for significant autocorrelation between measurements among goats. The statistical analyses were performed on the $\Delta\Delta Ct$ values and then converted to n-fold ($2^{-\Delta\Delta Ct}$) for data presentation. All values were presented as the least square means \pm SD. Differences were considered to be significant when $P < 0.05$.

RESULTS

Hematology examination

Total leukocyte and leukocyte differential counts were made before and after bacterial inoculation. The two counts significantly increased at 6 and 12 hr pi in experimental group goats and remained within normal ranges in the control over time points (Fig. 1a–1d).

Cervical cytology examination

Cytological examination of goats infused with *E. coli* revealed abundant purulent cervical discharge and a significant increase in the weight of the discharge at 3 hr pi, peaking at 6 hr pi (Fig. 2 Aa) and in %PMNs at 3 hr pi (83.56 \pm 5.95%) sustaining for 24 hr (Fig. 2 Ab), with no evident changes observed in the control group goats (Fig. 2A). The smears showed normal and intact epithelial cells (ECs) before inoculation (Fig. 2Ba), and abundant PMNs infiltrate after bacterial infusion at 3 hr pi (Fig. 2Bb), a few young epithelial cells (YECs) at 24 hr pi (Fig. 2Bc) and more YECs at 168 hr pi (Fig. 2Bd).

Electron microscope

It was observed in the experimental goats that the cobblestone-shaped endometrial epithelial cells were linked to each other and the surface of the epithelium was covered by integral microvilli (Fig. 3a) before bacterial infusion. Parts or even all microvilli were lost from the epithelium, and there were some discharge and cell debris that adhered to the surface of epithelial cells (Fig. 3b–3c) at 3 and 6 hr pi; the microvilli began to grow at 168 hr pi (Fig. 3d). However, no evident changes in the epithelial microvilli were seen before or after PBS infusion in the control goats (Fig. 3e–3f).

In the experimental goats, the endometrial epithelial cells were closely aligned and had oval nuclei prior to *E. coli* infusion (Fig. 4a), and parts of the microvilli (Mv) became shortened or were lost from the mitochondrial (Mi)-rich epithelial cells (Fig. 4b–4f) at 3 hr pi. Some inflammatory cells, such as neutrophils and lymphocytes, infiltrated the uterine glands (Fig. 4d) and lamina propria (Fig. 4e), some endometrial stromal cells degenerated with turgid and rounded nuclei (N) (Fig. 4e), and mast cell degranulation with some holes could be observed at 6 hr pi (Fig. 4f). In addition, the endometrial epithelial cell mitochondrial swelling with fractured cristae and a reduced number of desmosomes were observed at 12 hr pi (Fig. 4g). However, the endometrial epithelial cells retained integrity with no evident changes before or after PBS infusion (Fig. 4h–4i) in the control group goats.

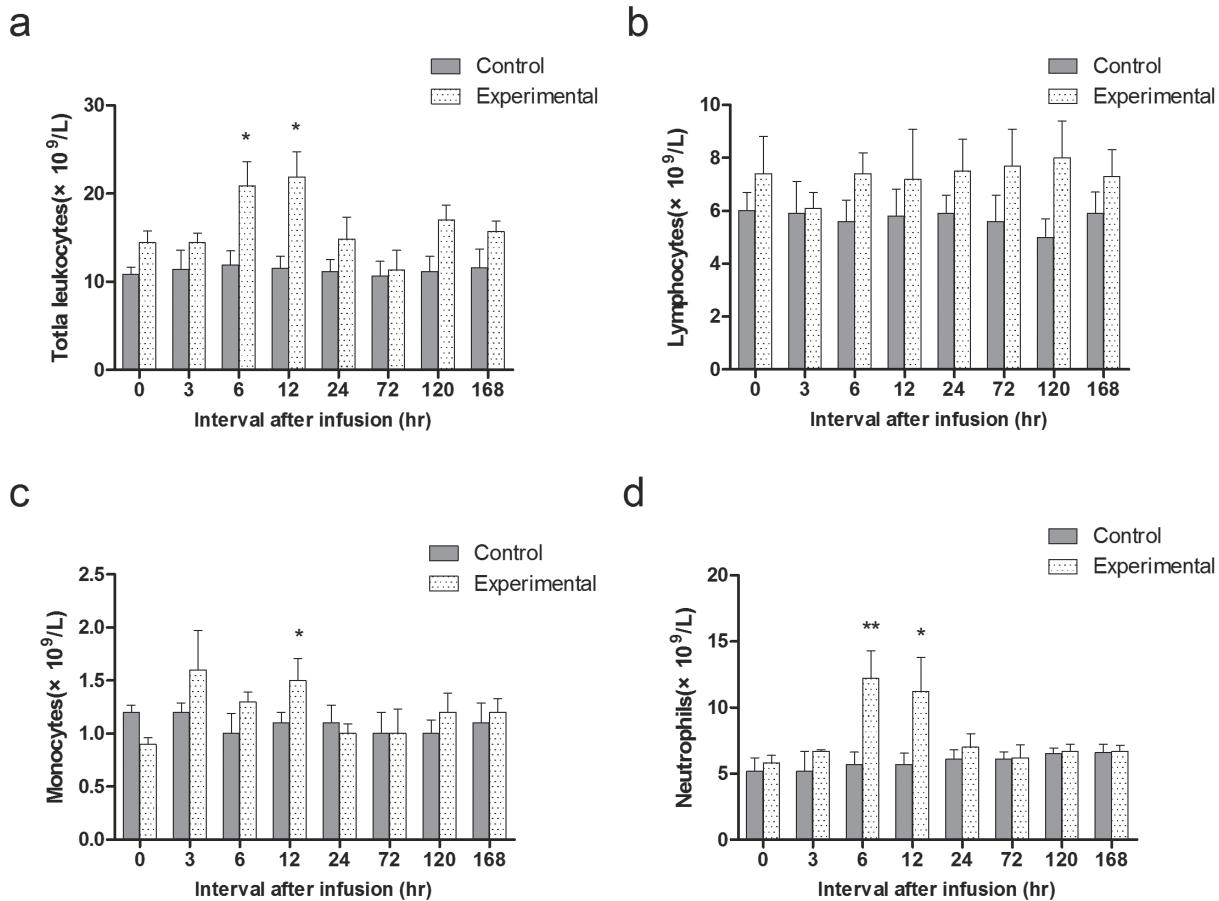


Fig. 1. (a) Total leukocyte, (b) lymphocyte, (c) monocyte and (d) neutrophil counts in goats infected with *E. coli* (experimental group, n=6) or treated with PBS (control group, n=6) compared to the pre-inoculation counts. The values represent means \pm SD. *, **P compared to pre-inoculation in the same group, *P<0.05, **P<0.01.

Expression of TLR4, β -defensin-2 and pro-inflammatory cytokines mRNA transcripts in cervical discharge

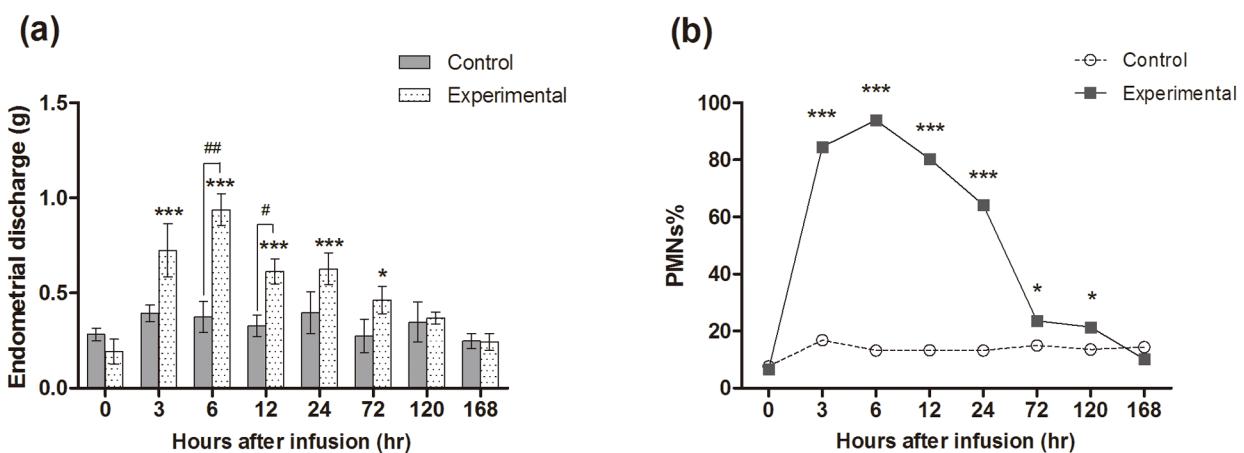
Intrauterine infusion with *E. coli* elicited a significant up-regulation of mRNA expression of TLR4, β -defensin-2 and cytokines in cervical discharge, whereas there was no significant change in samples from control goats (Fig. 5). There was a rapid significant increase in mRNA levels of TLR4 in samples from the experimental group at 3 hr pi, peaking (a 200-fold increase) by 12 hr pi ($P<0.001$; Fig. 5a) as compared with the pre-inoculation levels. In the experimental goats, the mRNA expression levels of a few factors in the cervical discharge significantly increased starting at 3 hr pi, with TNF- α peaking at 3 hr pi, a 50-fold; IL-1 β at 3 hr, a 4-fold; IL-6 at 12 hr, a 12-fold; IL-8 at 12 hr, a 7-fold; and β -defensin-2 at 12 hr, a 3-fold, compared with the pre-inoculation levels (Fig. 5b–f). By contrast, there were no significant changes in TNF- α , IL-1 β , IL-6, IL-8 or β -defensin-2 expression in control goats.

DISCUSSION

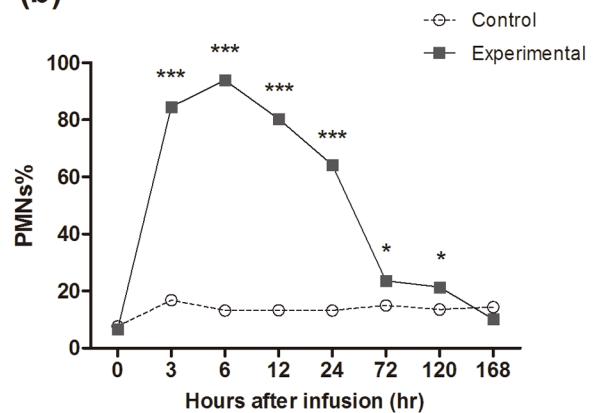
In a current study, the transient elevation of leukocytes in peripheral blood and the infiltration of PMNs into the uterine lumen were observed shortly after uterine bacterial infusion in goats, indicating a rapid activation of innate immune response. It was also noted that the %PMNs significantly increased at 3 hr pi, peaking at 6 hr pi and returning to pre-inoculation levels at 168 hr pi, which represents the initiation, development and elimination of uterine infection [20]. Besides the inflammatory cells, mature and young epithelial cells were also detected by the smears of cervical cytobrush samples. A few young endometrial cells with round nuclei and distinct cytoplasm were observed after *E. coli* inoculation in experimental goats, suggesting an endometrial recovery process [5]. All these cytology and hematology results together indicate a successful establishment of the subclinical endometritis model in goats.

We found that the results of cytological examination were consistent with the changes of endometrium epithelial cells observed by SEM and TEM. When the %PMNs significantly increased at 3–24 hr pi shown by the cervical cytology, the SEM examination revealed that the disappearance of parts of or all microvilli lost from the epithelial cells, and the TEM examination presented the infiltration with a number of neutrophils and monocytes appearing in the uterine glands and lamina propria. As demonstrated by electron microscopy, the microvilli began to grow at 3 day pi along with the decline of the %PMNs and the appearance of young endometrial cells in the experimental group goats, indicating that the %PMNs and the cellular morphous observed through

A



(b)



B

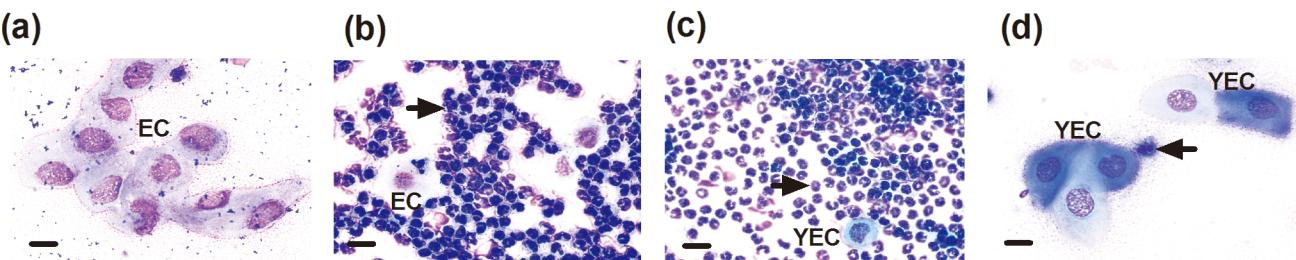


Fig. 2. The cervical discharge samples were collected using a sterile cytobrush and stained with modified Wright–Giemsma after air-drying. (A a) The weight of the cervical discharge in goats inoculated with PBS (Control: n=6) or *E. coli* (Experimental: n=6). *P<0.05, ***P<0.001. #, ##P compared to the control goats at the same sampling instant, #P<0.05, ##P<0.01. (A b) The %PMN in cervical discharge samples after intrauterine infusion with *E. coli* (Control: n=6) or PBS (Experimental: n=6). *P<0.05, ***P<0.001; (B a–d) The cellular morphous obtained at various intervals [(a) 0 hr, (b) 3 hr, (c) 24 hr and (d) 168 hr] after infusion with *E. coli*. The normal and intact epithelial cells (ECs) were obtained from cytobrush before bacterial infusion, abundant PMNs (arrows) infiltration at 3 hr and 24 hr pi and the appearance of a few young epithelial cells (YECs) at 24 hr and more YECs at 168 hr pi. Bar=10 μm.

the cytobrush smear reflected the endometrial status. Similar results were also reported by Bahareh [2] and Piotr [17]. We also found that the expression levels changes of pro-inflammatory genes tested in cervical discharge samples were in accordance with cytological changes. When the weight of cervical discharge and %PMNs significantly increased at 3–24 hr pi, these genes were significantly up-regulated by folds nearly at the same time. mRNA expression levels of TLR4 and other pro-inflammatory cytokines and AMPs significantly increased shortly after inoculation with *E. coli* in the cervical discharge. TNF-α is an activator of the transcription factor NF-κB and promotes a wide range of pro-inflammatory cytokine mRNA expression [6]. Endotoxin and other microbial products are strong stimuli for IL-1β transcription and then for the initiation of inflammatory cascade [3]. IL-6 is a pro-inflammatory cytokine produced during the early stages of inflammation with a broad range of activities, including PMNs maturation and activation [13]. IL-8 is one type of CXC chemokines, which primarily induces neutrophils migration from the circulation into a site of infection [18]. The expression of human β-defensin-2 is increased following stimulation by pro-inflammatory cytokines, and it primarily targets Gram-negative bacteria [25]. These may explain the rapid gene expression increase of TLR4, pro-inflammatory cytokines and AMPs in goats immediately after *E. coli* inoculation.

Some researchers argue that a low degree of agreement existed between the diagnostic outputs of uterine biopsy and cytology [16]. However, several studies have compared sensitivity and specificity between the endometrial biopsy and cytological brush for the diagnosis of endometritis in large animals and reported high agreement between endometrial cytology and uterine biopsy [1, 2, 9]. In addition, histopathological examination usually refers to paraffin sections and hematoxylin-eosin staining, but few studies looked at the endometrial ultrastructure in uterine infection animals, which might also explain the difference. We thus postulate that the difference on cytology and histology reported by different researchers may be due to species and procedures, which may grant further studies.

Clinically, cytology examination is restricted in practice to confirm uterine inflammation, and histopathology is usually considered the more accurate method to detect, define and classify endometritis [4]. However, cytological examination was also recommended to diagnose endometritis and monitor the elimination of inflammatory cells after treatment in cows [14] and mares [7]. In addition, the expression features of pro-inflammatory cytokines may serve as a further confirmation of clinical infection, but

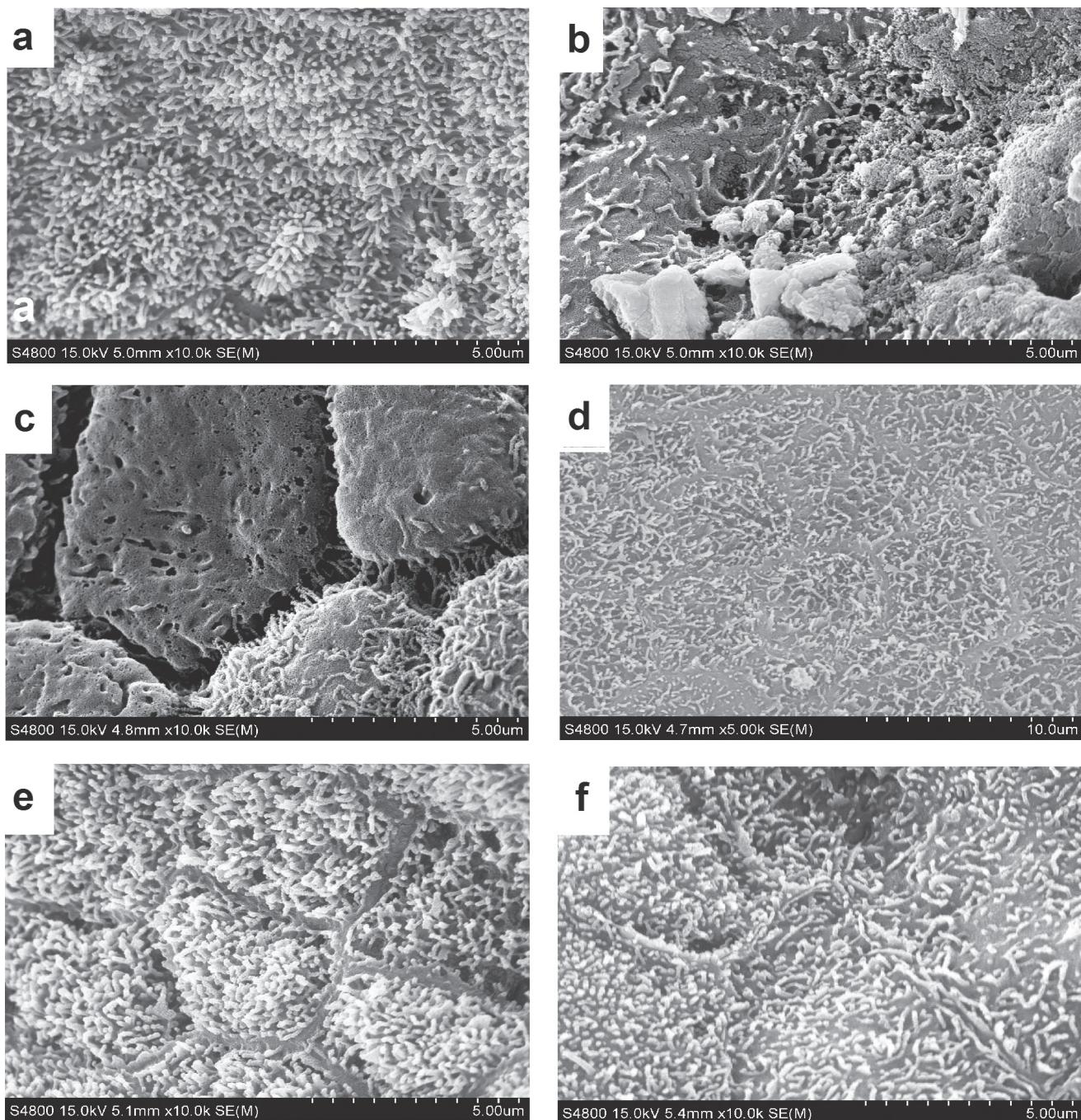


Fig. 3. Scanning electron microscopy examination of endometrial biopsy samples infused with *E. coli* [(a) 0 hr, (b) 3 hr, (c) 6 hr and (d) 168 hr] and PBS [(e) 0 hr and (f) 3 hr]. (a, e) The endometrial epithelial cells were covered with intact microvilli. (b, c) Some or all microvilli were lost from the epithelium, and some discharge and cell debris adhered to the surface of epithelial cells. (d) The microvilli began to grow at 168 hr pi in the *E. coli*-inoculated goats. (f) No evident inflammatory changes were observed after intrauterine infusion with PBS at 12 hr pi.

the examination on endometrial biopsy samples harms the reproductive system in cattle [10].

We conclude that the evaluation of cervical cytology was in a good accordance with molecular changes of cytokines in cervical discharge, hematology and histology. This novel, simple cervical cytobrush method can effectively obtain cervical discharge samples for cytology examination in the early diagnosis of subclinical endometritis in goats.

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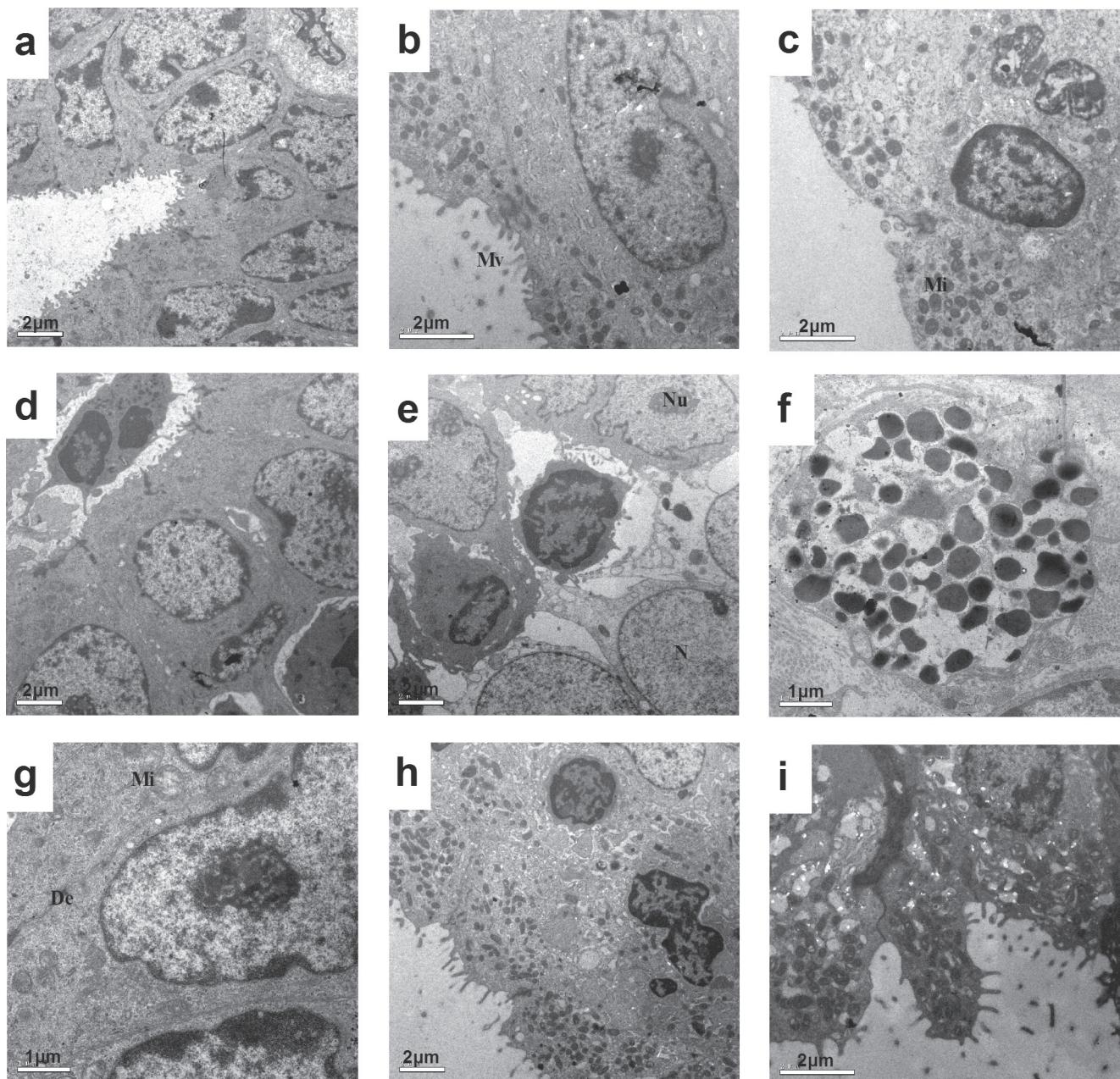


Fig. 4. Transmission electron microscopy examination of endometrial biopsy samples infused with *E. coli* [(a) 0 hr, (b–f) 3 hr and (g) 12 hr] or PBS [(h) 0 hr and (i) 3 hr]. (a) The endometrial epithelial cells were closely aligned and had oval nuclei. (b, c) Some of the microvilli (Mv) became shortened or were lost from the mitochondrial rich epithelium cells. (d, e) Some inflammatory cells, such as neutrophils and lymphocytes, infiltrated the uterine glands and lamina propria, and some endometrial stromal cells degenerated with turgid and rounded nuclei (N). (f) Mast cell degranulation, causing some holes, at 6 hr pi. (g) Endometrial epithelial cell mitochondrial swelling with fractured cristae and a reduced number of desmosomes were observed at 12 hr pi in the *E. coli*-infused goats. (h, i) The endometrial epithelial cells retained integrity with no evident changes before or after PBS infusion in control group goats.

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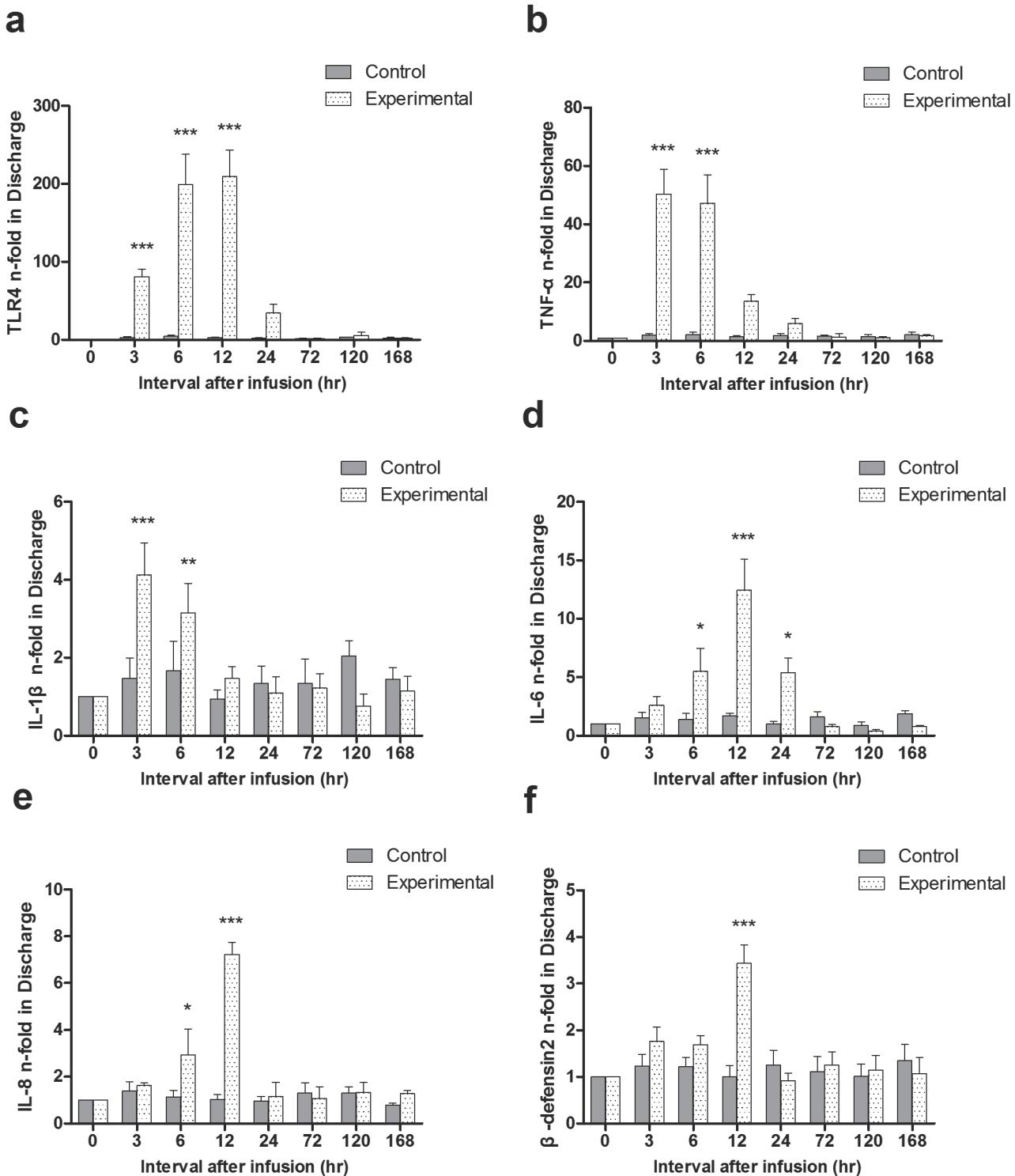


Fig. 5. Fold changes (n-fold) in (a) *TLR4*, (b) *TNF-α*, (c) *IL-1β*, (d) *IL-6*, (e) *IL-8* and (f) β -defensin-2 mRNA expression levels in cervical discharge of goats infected with *E. coli* (experimental group, n=6) or treated with PBS (control group, n=6). *, **, ***P compared to pre-inoculation in the same group, *P<0.05, **P<0.01, ***P<0.001.

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