Decreasing of S100A4 in bovine endometritis in vivo and in vitro

Yajuan Li, Dongsheng Wang, Xiaohu Wu, Baoxiang He, Zhangrui Cheng, Ottó Szenci, Pengjie Song, Dan Shao, Shidong Zhang, Zuoting Yan

PII: S0093-691X(20)30299-5

DOI: https://doi.org/10.1016/j.theriogenology.2020.05.015

Reference: THE 15521

To appear in: *Theriogenology*

Received Date: 8 June 2019

Revised Date: 10 May 2020

Accepted Date: 10 May 2020

Please cite this article as: Li Y, Wang D, Wu X, He B, Cheng Z, Szenci Ottó, Song P, Shao D, Zhang S, Yan Z, Decreasing of S100A4 in bovine endometritis in vivo and in vitro, *Theriogenology* (2020), doi: https://doi.org/10.1016/j.theriogenology.2020.05.015.

Author Contribution

(1)the conception and design of the study: Shidong Zhang, Zuoting Yan

(2)acquisition of data: Yajuan Li, Dongsheng Wang, Xiaohu Wu, Pengjie Song, Dan Shao

(3) analysis and interpretation of data: Yajuan Li, Shidong Zhang

(4)drafting the article or revising it critically for important intellectual content: Yajuan Li, Shidong Zhang, Zuoting Yan, Baoxiang He, Zhangrui Cheng, Otto. Szenci

(5)final approval of the version to be submitted: Shidong Zhang, Zuoting Yan

"revised"

Decreasing of S100A4 in bovine endometritis in vivo and in vitro

Yajuan Li [a, b], Dongsheng Wang [a], Xiaohu Wu [a], Baoxiang He [b], Zhangrui Cheng [c], Ottó Szenci [d], Pengjie Song [a], Dan Shao [a], Shidong Zhang [a, *], Zuoting Yan [a, *]

[a] *Key Laboratory of Veterinary Pharmaceutical Development of Ministry of Agriculture, Lanzhou Institute of Husbandry and Pharmaceutical Sciences of CAAS, Lanzhou 730050, P. R. China*

[b] *College of Animal Science and Technology, Guangxi University, Nanning 530005, P. R. China*

[c] *Department of Pathobiology and Population Sciences, Royal Veterinary College, Hertfordshire AL9 7TA, UK*

[d] *MTA-SZIE Large Animal Clinical Research Group, University of Veterinary Medicine Budapest, Üllő H-2225, Hungary)*

Declarations of interest: none

[*]Corresponding author. Key Laboratory of Veterinary Pharmaceutical Development of Ministry of Agriculture, Lanzhou Institute of Husbandry and Pharmaceutical Sciences of CAAS, Lanzhou 730050, P. R. China
E-mail address: sdzhang2006@126.com (Shidong Zhang), yanzuoting@caas.cn (Zuoting Yan)

Abstract

Endometritis is a prevalent reproductive disease in dairy cows, and is a superficial inflammation of the endometrium. S100 calcium-binding protein A4 (S100A4) is suggested to be implicated in the progression of inflammation. However, to our knowledge, no study has reported the changes of S100A4 during bovine endometritis. The objective of this study was to investigate S100A4 gene expression and protein levels in the uterus with endometritis in dairy cows. Vaginal mucus samples were collected for diagnosis of the severity degree of endometritis and the detection of S100A4 protein content. Blood samples and endometrial biopsies were collected and divided into the control (CN), mild endometrtis (M), and severe endometritis (S) groups according to the characteristics of the vaginal mucus type. The isolated bovine endometrial epithelial cells (BEECs) were challenged with *E. coli* (2×10^6 CFU/mL, 2×10^7 CFU/mL) or lipopolysaccharide (LPS, 3 and 10 μg/mL) as an inflammatory model. RT-qPCR was used to detect the gene expression levels of S100A4 and cytokines, including interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-10 (IL-10), and tumour necrosis factor-alpha (TNF-α), in tissues or cells. Enzyme-linked immunosorbent assay (ELISA) was used for S100A4 protein level detection in tissues, cells, cell supernatant, vaginal mucus, and serum samples. The results showed that S100A4 gene and protein levels decreased in bovine endometrium with endometritis and in *E. coli*- or LPS-stimulated BEECs. We failed to detect S100A4 in the cell supernatant, vaginal mucus, and serum samples. This study suggested that S100A4 is a pathogenesis-related protein of endometritis, and

decreased expression of S100A4 may pave the way for the development of endometritis in dairy cows.

Key words: S100A4, endometritis, dairy cow

1. Introduction

As a result of milk accounting for a large proportion of the human diet, the health conditions of dairy cows have attracted great attention. Reproductive disease, especially endometritis, is one of the most important diseases compromising animal welfare and causing increased economic loss in the dairy industry [1]. Endometritis is highly prevalent in dairy cows and can result in other kinds of reproductive diseases, including pyometra, anovulation and pregnancy loss, as well as reduce productive performance [2, 3].

S100 calcium-binding proteins are widely reported to be crucial to many diseases and are considered potential biomarkers in the diagnosis and treatment of several diseases [4]. S100A4 is an S100 protein family member that functions in both intracellular and extracellular pathways[5]. Existing studies have shown that S100A4 protein is expressed in immune cells, such as monocytes, macrophages, and polymorphonuclear granulocytes, and is also expressed at low levels in normal tissues [6, 7]. S100A4 plays important roles in a range of biological functions, including cell adhesion, movement, invasion, and metastasis [8]. Numerous studies on S100A4 have focused on tumours and cancer [5]. S100A4 has also been reported tobe involved in inflammation progression. For example, S100A4 can aggravate the clinical symptoms of colitis in mice [9], and it may be a potent trigger of inflammatory processes and

induce the release of cytokines [10]. It has also been reported that S100A4 promoted the progression of endometritis, while deficiency of S100A4 reduced the mRNA expression levels of IL-1β and TNF-α in the endometrium of mice [11]. However, the roles of S100A4 in bovine endometritis have not been reported. To investigate the roles of S100A4 in endometritis of dairy cows, we determined the gene and protein expression of S100A4 in endometrial tissues, vaginal mucus, serum, *E. coli*/LPS-stimulated endometrial epithelial cells, and cell supernatant.

2. Materials and methods

2.1. Animals and diagnostic samples

We randomly selected twenty-two Holstein-Friesian cows, aged 3 to 5 years and 2 to 3 parities on 40 to 60 days postpartum without systemic symptoms. Vaginal discharge samples were collected using a draw-tube. All cows were divided into three groups based on discharge detection: control group (CN, n=6) characterized by clear vaginal mucus, mild endometritis group (M, n=8) characterized by less than 50% white or off-white mucopurulent material in vaginal discharge, and severe endometritis group (S, n=8) characterized by more than 50% white or yellow purulent material in vaginal discharge [12]. Endometrial biopsy samples were obtained using sterile uterine biopsy punch forceps from the right side of the uterine horn. The tissue samples were divided into two parts: one was immediately placed into liquid nitrogen for total protein and RNA extraction, and another was placed into 10% neutral formalin for histopathology using routine haematoxylin and eosin (HE) staining.

Bovine endometrial epithelial cells (BEECs) were isolated from a healthy uterus of a six-month old dairy cow. Briefly, a healthy uterus was brought to the laboratory in a sterile PBS (pH 7.2) containing penicillin (100 μg/mL) and streptomycin (100 U/mL). Endometrium from the uterine horn was cut off into 2–3 cm long pieces, washed in PBS (pH 7.2) twice, and then uterine tissue was digested with 1% collagenase I (Sigma, USA) diluted in DMEM/F12 (HyClone, USA) for 6 h. The digested endometrium was scraped using a sterile cell scraper, and scraped materials were collected and washed in PBS (pH 7.2). Then, the collected materials were centrifuged at 100 × g for 5 min to collect the cell suspension. Trypan Blue stain was used to estimate cell viability. Cells were cultured in DMEM/F12 with 10% foetal bovine serum (Gibco, Australia), penicillin (100 μg/mL), and streptomycin (100 U/mL) at 37 °C with 5% CO2 and 95% sterile air when the viability >95%. The medium was changed every 3 days until the cells reached approximately 90% confluence.

Considering that the cultures were inevitably mixed with some stromal cells, we removed stromal cells according to the different sensitivities of epithelial cells and stromal cells to trypsin [13]. We conducted time-different digestion thrice to obtain purified epithelial cells. We used the expression of epithelial-specific cytokeratin to estimate the purification of epithelial cells using a mouse monoclonal antibody (Abcam, ab49779) and goat anti-mouse IgG-FITC (Abcam, ab6785).

Cells growing in logarithmic phase were inoculated into a six-well plate at a concentration of 2×10^5 cells/well, and incubated for 24 h as described above. They were then treated with different concentrations of *E. coli* (2×10^6 CFU/mL, 2×10^7

CFU/mL) for 2 h or LPS (O111:B4, Sigma) at 0, 3, or 10 μg/mL for 24 h. After stimulation, the cells and supernatant were collected for gene or protein analysis of S100A4. Three biological replicates and three technological replicates were included.

2.2. Diagnostic assays

Total RNA was extracted from BEECs or endometrial biopsy tissues using RNAiso Plus (Takara, Japan), and 600 ng of RNA was used for reverse transcription by the PrimeScript RT reagent Kit with gDNA Eraser (Takara, Japan). RT-qPCR was performed using SYBR Premix Ex Taq □ (Takara, Japan) on an iQ5 thermal cycler (Bio-Rad, USA). The fold change in mRNA expression relative to β-actin was calculated using the $2^{-\Delta\Delta Ct}$ method. The involved primers are detailed in Table 1.

A commercial kit (Transgen, China) was used for total protein extraction in endometrial biopsies and cells. Briefly, grounding tissues (ground with liquid nitrogen using a mortar) and cells were treated with lysis solution to release total protein. Then, the samples were centrifuged at 14,000×g for 10 min at 4°C to collect total protein. The concentration of total protein was measured using a BCA Protein Assay Kit (Takara, Japan).

We used a commercial ELISA kit for human beings (CUSABIO, China) to determine the S100A4 level in total protein samples from tissues and cells, cell supernatant, vaginal mucus, and serum samples. The detection range of the S100A4 contents using this ELISA kit is 0.9 ng/mL to 60 ng/mL. MULTISKAN MK3 (Thermo, USA) was used to read the plate. Serum was isolated from blood samples by centrifugation at 3000×g for 10 min. Pre-treatment of vaginal mucus was performed

before ELISA detection. The mucus was diluted with sterile PBS containing protease inhibitor, then centrifuged at 12,000×g for 10 min. A BCA Protein Assay Kit (Takara, Japan) was used to measure the concentration of total protein in mucus.

2.3. Statistical analysis

Statistical data analysis was carried out with analysis of variance built in SPSS 19 (Chicago, IL, USA). All data are presented as means±SD. One-way ANOVA analysis was used to compare the results among groups. Statistical significance was considered at $P<0.05$. Differences among all groups at each index were performed. Totally different letters indicated significant difference between two group ($P<0.05$).

3. Results

As shown in Fig. 1, the healthy endometrium tissues had a complete epithelium composed of normal epithelial cells; in mild endometritis tissues, the epithelial cells were arranged irregularly in the endometrium with prominent lymphocyte-rich infiltration and low-grade plasmocyte infiltration; the endometrium tissues with severe endometritis showed lymphocyte migration, haemorrhage, and epithelial necrosis. The histopathological features of the three groups were consistent with vaginal mucus classification. The gene and protein expression of S100A4 in the classified tissue samples was detected, and the results showed that both the gene expression level and protein content of S100A4 were significantly decreased in endometrial tissues with mild or severe endometritis (Fig. 2) and negatively correlated with the severity of endometritis.

The results of the in vitro experiment indicated that, compared with normal BEECs, TNF-α, IL-1β, IL-6, and IL-10 were upregualted significantly when BEECs were stimulated with *E. coli* (2×10^6 CFU/mL, 2×10^7 CFU/mL), with the exception of IL-6 levels after 2×10^6 CFU/mL *E. coli* stimulation (Fig. 3A-D). Likewise, the mRNA levels of TNF-α, IL-1β, IL-6 and IL-10 increased significantly when BEECs were treated with 3 or 10 μg/mL LPS (Fig 3E-H). Nevertheless, S100A4 gene levels significantly decreased in BEECs treated with *E. coli* (2×10^6 CFU/mL, 2×10^7 CFU/mL) or LPS (3, 10 μg/mL), and protein levels also significantly decreased in BEECs treated with 2×10^7 CFU/mL *E. coli*. Interestingly, S100A4 protein decreased without a significant difference after LPS treatment (Fig. 4). Additionally, S100A4 protein was not detected in vaginal discharge, serum, and cell supernatant by ELISA.

4. Discussion

Many S100 calcium-binding protein family members have potent antimicrobial properties and are essential components of the immune response to invading pathogens during infection and inflammation [10]. For example, S100A12 can promote vascular and valve calcification to regulate vascular inflammation in non-oxidized low-density lipoprotein-induced vascular smooth muscle cells [14]. Exogenous S100A12 significantly upregulated MMP-13 and VEGF via the p38 MAPK and NF-κB pathways [15], and high expression of S100A8/9 was observed in osteoarthritis [16].

171 While some evidences strongly support S100A4 as an inflammation-related
172 protein, the regulatory mechanism of S100A4 is unclear [10]. One study reported that
173 S100A4 was upregulated in LPS-treated endometritis, and a lack of S100A4 resulted
174 in an impaired inflammatory response in mice models of acute endometritis [11].
175 S100A4 has also been reported to induce a network of inflammatory cytokines or
176 promote the inflammatory response in mononuclear cells [17]. However, another
177 study found that S100A4 decreased the release of cytokines in a high fat-diet
178 inflammatory model [18]. Therefore, the inflammatory regulation of S100A4 may be
179 different under various conditions, suggesting that the relative mechanisms still need
180 further study.

181 Bovine endometritis often occurs postpartum within 60 days. However, the
182 alteration of S100A4 in bovine endometritis is unknown. In this study, we chose cows
183 40to 60 days postpartum that experienced the prolonged uterine inflammation [12].
184 We first found that S100A4 mRNA expression levels and protein contents decreased
185 in bovine endometrium with endometritis (Fig. 2), but increased in some reports [9,
186 11]. Furthermore, combined with pathological grade, S100A4 decrease was closely
187 related to the intensity of inflammation in bovine endometrium (Fig. 1 and Fig.2).
188 Thus, decreased S100A4 may contribute to deterioration of endometritis in dairy
189 cows.

190 Because endometritis is an inflammatory response of endometrial epithelial cells,
191 an inflammatory cell model should be used to research S100A4. We successfully
192 developed a cell model using *E. coli* or LPS stimulation and evaluated it by cytokine

193 detection (Fig. 3). In vitro results showed that cytokines increased with the dosage of
194 bacteria or LPS, however, S100A4 was negatively correlated with the dosage of *E.*
195 *coli* or LPS in BEECs (Fig. 4). Thus, the decrease of S100A4 was also closely related
196 to the intensity of cellular inflammation in vitro. Although these results were not in
197 agreement with other studies [17], we deem that bovine S100A4 may be a particular
198 factor that can be downregulated during bovine endometritis. Additionally, S100A4
199 protein could not be detected in vaginal discharge samples and cell supernatant by
200 ELISA. This may result from bovine S100A4 being an intracellular protein located at
201 the perinuclear region of the cytoplasm (https://www.uniprot.org/uniprot/P35466),
202 which is different from other S100A members that are secreted as extracellular
203 proteins. Meanwhile, it has to point out that we used a commercial human ELISA kit
204 to detect bovine S100A4 content because both proteins have 96.04% similarity in
205 amino acid sequence (P26447 and P35466 in the Uniprot Database). Although the
206 result is deduced, to a large extent, bovine S100A4 alteration is closely related to
207 endometritis progression.

208 In conclusion, the decrease of intracellular S100A4 was closely related to
209 endometritis in dairy cows, and S100A4 is a pathogenesis-related protein of
210 endometritis. We tentatively proposed that decreased expression of S100A4 may play
211 an important role in the development of bovine endometritis. However, the function
212 and mechanism of S100A4 in bovine endometritis still need further investigation.

213 **Ethics statement**

The present study was approved by the Institutional Animal Care and Use Committee of Lanzhou Institute of Husbandry and Pharmaceutical Sciences of Chinese Academy of Agricultural Sciences (SYXK-2014-0002).

Acknowledgments

This work was financially supported by grants 13th five-year plan of National Key Research and Development Program of China (No.2017YFD0502201) and Science and Technology Innovation Project of Chinese Academy of Agricultural Sciences (NO. CAAS-ASTIP-2014-LIHPS-03).

References

[1] Gilbert RO. Management of reproductive disease in dairy cows. Vet Clin North Am Food Anim Pract 2016;32: 387-0. https://doi.org/10.1016/j.cvfa.2016.01.009.

[2] Santos JE, Bisinotto RS, Ribeiro ES, Lima FS, Greco LF, Staples CR, et al. Applying nutrition and physiology to improve reproduction in dairy cattle. Soc Reprod Fertil Suppl 2010;67: 387-3. https://doi.org/10.7313/upo9781907284991.030.

[3] Dubuc J, Denis-Robichaud J. A dairy herd-level study of postpartum diseases and their association with reproductive performance and culling. J Dairy Sci 2017;100: 3068-8. https://doi.org/10.3168/jds.2016-12144.

[4] Ambartsumian N, Klingelhofer J, Grigorian M. The Multifaceted S100A4 Protein in Cancer and Inflammation. Methods Mol Biol 2019;1929:339-5. https://doi.org/10.1007/978-1-4939-9030-6_22.

[5] Fei F, Qu J, Zhang M, Li Y, Zhang S. S100A4 in cancer progression and metastasis: A systematic review. Oncotarget 2017;8: 73219-9. https://doi.org/10.18632/oncotarget.18016. eCollection 2017 Sep 22.

[6] Ilg EC, Schafer BW, Heizmann CW. Expression pattern of S100 calcium-binding proteins in human tumors. Int J Cancer 1996;68:325-3. https://doi.org/10.1002/(SICI)1097-0215(19961104)68:3<325::AID-IJC10>3.0.CO;2-7.

[7] Takenaga K, Nakanishi H, Wada K, Suzuki M, Matsuzaki O, Matsuura A, et al. Increased expression of S100A4, a metastasis-associated gene, in human colorectal adenocarcinomas. Clin Cancer Res 1997;3:2309-16.

244 [8] Mishra SK, Siddique HR, Saleem M. S100A4 calcium-binding protein is key
245 player in tumor progression and metastasis: preclinical and clinical evidence. Cancer
246 Metastasis Rev 2012;31: 163-172. https://doi.org/10.1007/s10555-011-9338-4.
247 [9] Zhang J, Hou S, Gu J, Tian T, Yuan Q, Jia J, et al. S100A4 promotes colon
248 inflammation and colitis-associated colon tumorigenesis. Oncoimmunology 2018;7:
249 e1461301. https://doi.org/10.1080/2162402X.2018.1461301. eCollection 2018.
250 [10] Zackular JP, Chazin WJ, Skaar EP. Nutritional Immunity: S100 Proteins at the
251 Host-Pathogen Interface. J Biol Chem 2015;290: 18991-8.
252 https://doi.org/10.1074/jbc.R115.645085. Epub 2015 Jun 8.
253 [11] Wu Y, Zhang J, Qin Y. S100A4 promotes the development of
254 lipopolysaccharide-induced mouse endometritis. Biol Reprod 2018; 99:960-967.
255 https://doi.org/10.1093/biolre/ioy124.
256 [12] Williams EJ, Fischer DP, Pfeiffer DU, England GC, Noakes DE, Dobson H,
257 Sheldon IM. Clinical evaluation of postpartum vaginal mucus reflects uterine
258 bacterial infection and the immune response in cattle. Theriogenology 2005;63:
259 102-117. https://doi.org/10.1016/j.theriogenology.2004.03.017.
260 [13] Fortier MA, Guilbault LA, Grasso F. Specific properties of epithelial and stromal
261 cells from the endometrium of cows. J Reprod Fertil 1988;83: 239-248.
262 https://doi.org/10.1530/jrf.0.0830239.
263 [14] Chellan B, Sutton NR, Hofmann BM. S100/RAGE-Mediated Inflammation and
264 Modified Cholesterol Lipoproteins as Mediators of Osteoblastic Differentiation of
265 Vascular Smooth Muscle Cells. Front Cardiovasc Med 2018;5: 163.

https://doi.org/10.3389/fcvm.2018.00163. eCollection 2018.

[15] Nakashima M, Sakai T, Hiraiwa H, Hamada T, Omachi T, Ono Y, et al. Role of S100A12 in the pathogenesis of osteoarthritis. Biochem Biophys Res Commun 2012;422: 508-4. https://doi.org/10.1016/j.bbrc.2012.05.036. Epub 2012 May 16.

[16] Cremers NAJ, van den Bosch MHJ, van Dalen S, Di Ceglie I, Ascone G, van de Loo F, et al. S100A8/A9 increases the mobilization of pro-inflammatory Ly6Chigh monocytes to the synovium during experimental osteoarthritis. Arthritis Res Ther 2017; https://doi.org/19:217. doi: 10.1186/s13075-017-1426-6.

[17] Hansen MT, Forst B, Cremers N, Quagliata L, Ambartsumian N, Grum-Schwensen B,et al. A link between inflammation and metastasis: serum amyloid A1 and A3 induce metastasis, and are targets of metastasis-inducing S100A4. Oncogene 2015;34:424–5. https://doi.org/ 10.1038/onc.2013.568. Epub 2014 Jan 27.

[18]Hou S, Jiao Y, Yuan Q, Zhai J, Tian T, Sun K, et al. S100A4 protects mice from high-fat diet-induced obesity and inflammation. Lab Invest 2018;98:1025-1038. https://doi.org/ 10.1038/s41374-018-0067-y. Epub 2018 May 22.

281 **Table 1** Primer pairs used for q-PCR

Gene name	ID	Sequence
β-actin	NM_173979.3	F- CTCTTCCAGCCTTCCTTCCT
		R-GGGCAGTGATCTCTTTCTGC
S100A4	NM_174595.2	F- AAAGTGAAGGCTCCTCAGGTGT
		R- GTTCAGTATGGTGCTCACGTCT
TNF-α	NM_173966.3	F-TCCAGAAGTTGCTTGTGCCT
		R-CAGAGGGCTGTTGATGGAGG ・
IL-1β	NM_174093.1	F-CCTCGGTTCCATGGGAGATG
		R- AGGCACTGTTCCTCAGCTTC ・・
IL-6	NM_173923.2	F-GCTGAATCTTCCAAAAATGGAGG
		R-GCTTCAGGATCTGGATCAGTG ・
IL-10	NM_174088.1	F-CCTTGTCGGAAATGATCCAGTTTT
		R-TCAGGCCCGTGGTTCTCA ・・

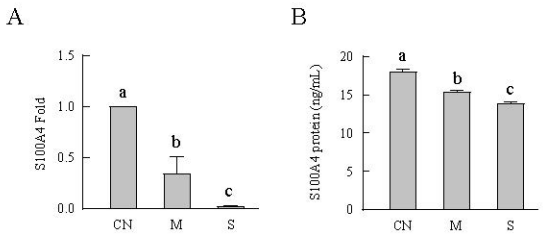
Figure legends

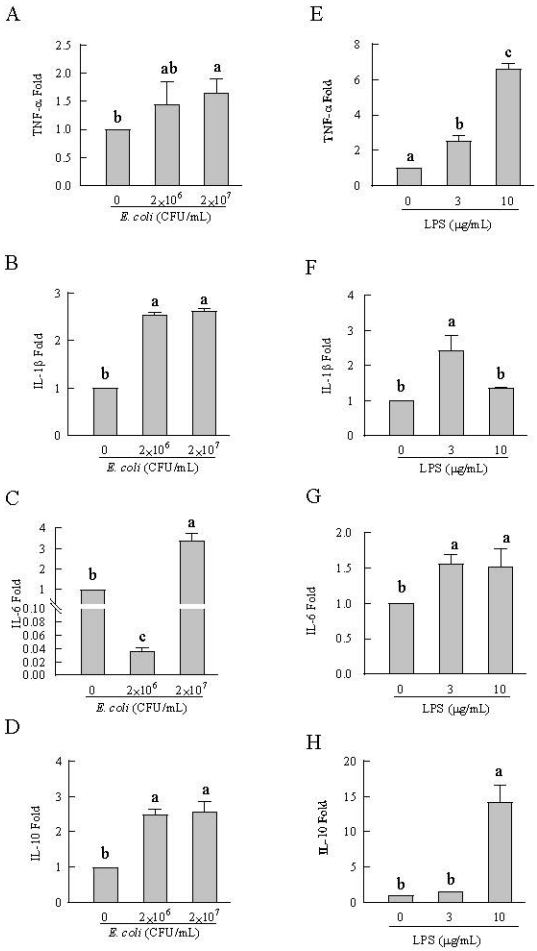
Fig. 1 Endometrial biopsy tissue sections from dairy cows stained with HE: CN, M, and S represents endometrial biopsy from healthy cows, cows with mild endometritis, and cows with severe endometritis, respectively. Cells labeled as E, epithelial cell; L, lymphocyte; P, plasmocyte; R, red blood cell. NE represents epithelial cells necrosis. Images were magnified 400×.

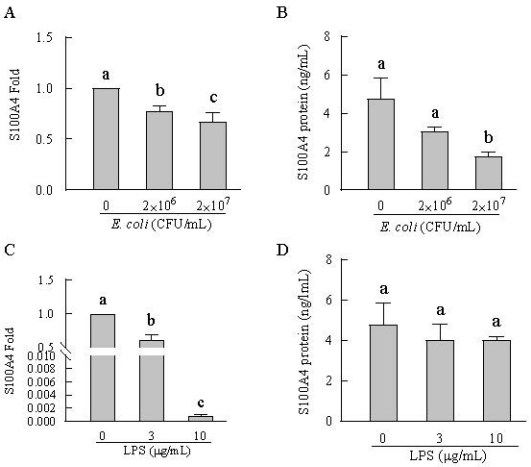
Fig. 2 Gene expression and protein content of S100A4 in endometrial tissue. Different letters mean significant difference, P<0.05. CN, M and S represented healthy endometrium, mild endometritis and severe endometritis endometrium, respectively.

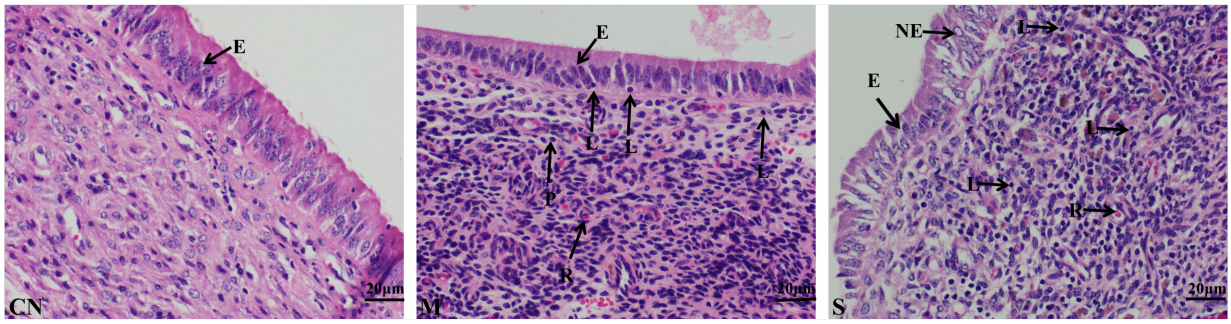
Fig.3 Gene expression of inflammatory factors in *E. coli* or LPS-stimulated BEECs. A-D: the expression of TNF-α, IL-1β, IL-6 and IL-10 in *E. coli* stimulated BEECs. E~H: the expression of TNF-α, IL-1β, IL-6 and IL-10 in LPS stimulated BEECs. Different letters mean significant difference, *P*<0.05.

Fig. 4 Gene expression and protein content of S100A4 in *E. coli* or LPS-stimulated BEECs. Different letters mean significant difference, *P*<0.05. A-B: the expression and protein contents decreased in *E. coli*-stimulated BEECs; C-D: the expression and protein contents decreased in LPS-stimulated BEECs.









1, This work firstly indicated that S100A4 decreased in endometritis of dairy cows in vivo and in vitro.