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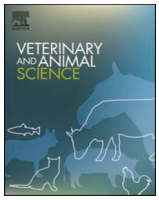

Inflammatory uterine microenvironment in long-term infertility repeat breeder cows compared with normal fertile cows

Maho Taru [a], Taiga Katoh [a], Karen Koshimizu [a], Sohei Kuribayashi [a], Ryotaro Miura [b], Seizo Hamano [c], Koumei Shirasuna [a,*]

[a] Department of Animal Science, Tokyo University of Agriculture, Kanagawa 243-0034, Japan
[b] Department of Veterinary Medicine, Nippon Veterinary and Life Science University, Tokyo 180-8602, Japan
[c] Artificial Insemination Association of Japan, Tokyo 135-0041, Japan

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ABSTRACT

The reproductive performance of lactating dairy cows is gradually declining, and one of the causes of this problem is the presence of long-term infertility repeat breeder cows (RBCs). The causes of RBCs are largely thought to be maternal factors, including the uterine environment. This study aimed to accurately investigate the uterine environment of RBCs using uterine tissue and fluid. Next, we investigated the effect of nobiletin in bovine endometrial epithelial cells to explore the possibility of improving the uterine environment of RBCs.

Uterine fluid was collected by flushing the uterus and endometrial tissues were collected by biopsy on day 7 of the estrous cycle from both normal fertile cows and RBCs ($n = 5$ in each group). A comprehensive analysis of the uterus revealed that gene expression and altered pathways differed between normal fertile cows and RBCs. Especially, pathways of natural killer cell-mediated cytotoxicity, cell cycle, and calcium signaling pathway were picked up in the uterine tissues of RBCs. In the uterine fluid, the levels of lipopolysaccharide were higher in the RBC than in normal group ($P = 0.08$). In in vitro experiment, treatment with the uterine fluid from RBCs upregulated inflammation-related pathways and molecules such as interleukin-8 (IL-8) in bovine endometrial epithelial cells. The treatment with nobiletin suppressed IL-8 induced by the treatment with uterine fluid.

In conclusion, the uterine environment of RBCs was found to be in inflammatory condition, causing the lower reproductive performance. It is necessary to develop methods to improve to the anti-inflammatory state in the uterine environment of RBCs.

1. Introduction

The reproductive performance and fertility of lactating dairy cows is gradually declining worldwide. Long-term infertility repeat breeder cows (RBCs) are subfertile animals without anatomical or infectious abnormalities that do not get pregnant after three or more breeding attempts (Dochi et al., 2008). On the other hand, 52.7 % of RBCs have subclinical endometritis; therefore, uterine infections, such as bacterial lipopolysaccharides (LPS), are important risk factors for a lesser reproductive performance in dairy cows (Heidari et al., 2019; Salasel et al., 2010). Repeat breeding causes an increase in insemination time and duration until the next pregnancy (days open), resulting in economic losses and costs associated with milk production for farmers (Perez-Marin & Quintela, 2023).

Many studies examined the factors that contribute to the repeat breeding syndrome (RBS). Recently, Perez-Marin and Quintela (2023) reviewed RBS and found that multiple factors contributed to its occurrence, including subclinical endometritis, nutritional conditions, abnormal estrous behavior, abnormal hormonal regulation (progesterone, lutenizing hormone, and epidermal growth factor), delayed ovulation, and lower oocyte and embryo quality (Bage et al., 2002; Canu et al., 2010; Katagiri & Moriyoshi, 2013). In particular, the cause of RBS is thought to be largely due to maternal factors, primarily the uterine environment (Perez-Marin & Quintela, 2023). In support of this possibility, Kimura et al. (2010) demonstrated that after the transfer of an elongating embryo, the rates of conception to pregnancy in RBCs were dramatically lower than those of their healthy fertile cows. We previously showed that embryo transfer following artificial insemination (AI) improves conception rate in RBCs (48.8 %, higher than pregnancy rate in RBCs (23.4 %) via normal AI) (Yaginuma et al., 2019). However, even

* Corresponding author.
E-mail address: ks205312@nodai.ac.jp (K. Shirasuna).

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with this technique, more than half of the RBCs became infertile. In other words, even if one of the maternal problems is eliminated by transferring a well-fertilized egg or good-quality embryo, improving the fertility of RBCs may be difficult.

Based on the above results, it is hypothesized that the environment of the uterus in RBCs differs from that in healthy normal cows and is likely to be less suitable for supporting successful embryo implantation. Indeed, endometrial gene expression profiles differed between RBCs and normal fertile cows (Hayashi et al., 2017; Hayashi & Sakumoto, 2023; Jaureguiberry et al., 2020; Kasimanickam et al., 2020, 2014). Moreover, intrauterine administration of seminal plasma, osteopontin, or platelet concentrates can increase the percentage of pregnancy in RBCs (Katagiri & Moriyoshi, 2013; Kyaw et al., 2022; Lange-Consiglio et al., 2015). These results suggest that it is important to appropriately regulate the uterine environment of RBCs for pregnancy.

The RBS remains largely unmanageable owing to lack of informative specific factors. Therefore, the first one of the present study was to accurately investigate the uterine environment of RBCs using uterine tissue and fluids. There is increasing evidence that citrus flavones, including nobiletin, have various beneficial role owing to their strong anti-inflammatory, anti-cancer, and anti-atherogenic activities (Ashrafizadeh et al., 2020). The second one was to explore the possibility of improving the uterine environment of RBCs, and for this purpose we focused on nobiletin and investigated it in bovine endometrial epithelial cells.

2. Materials and methods

2.1. Design of animal experiments and sampling

All experimental procedures complied with the guidelines for the care and use of agricultural animals of the Tokyo University of Agriculture, and all animal protocols were approved by the institutional animal experiment committee.

Normal fertile Holstein cows in the university farm ($n = 5$; parity 1–4; milk production, 28.1–44.3 kg/day) were controls. After normal parturition, estrus synchronization was performed using estradiol benzoate (ovahormon, 2 mg, intramuscular administration, Aska pharmaceutical, Tokyo, Japan) and progesterone formulations (controlled internal drug release [CIDR], Zoetis Japan, Tokyo, Japan). After 9 days, CIDR was removed and prostaglandin $F_{2\alpha}$ (dinoprost, 25 mg, intramuscular administration, Zoetis Japan) was injected to induce luteolysis, and gonadotropin-releasing hormone (Fertirelin acetate, 100 μg, intramuscular administration, Fujita pharmaceuticals, Tokyo, Japan) was injected to induce ovulation (day 1). After confirming ovulation by observation using ultrasound diagnostic, the endometrial tissues and uterine fluid were collected on day 7 of the estrous cycle. Average of sampling was 66.6 ± 3.0 days (59–74 days) after parturition. Subsequently, the cows were subjected to AI during spontaneous estrous cycle, and pregnancy was determined by observation using ultrasound diagnostic on 40 days after insemination. Individuals who conceived within three attempts of AI were considered normally fertile cows.

RBCs in the university farm were defined by following (Dochi et al., 2008; Funeshima et al., 2019): RBCs showed normal estrous behavior, normal uterus and ovaries as determined by observation using ultrasound diagnostic, and the inability to conceive after four or more AIs following normal estrous behavior ($n = 5$). Then, the estrous cycle was synchronized using the above method used for normal fertile cows. Endometrial tissues and uterine fluid were collected on day 7 of the estrous cycle. Average of sampling was 317.6 ± 50.4 days (209–479 days) after parturition.

To collect the uterine fluid, the uterine horns ipsilateral to the ovary with the corpus luteum were flushed individually using the embryo recovery technique on day 7 of the estrous cycle using a balloon catheter (Fujihira Industry, Tokyo, Japan), as previously described (Funeshima et al., 2021). The collected flushing fluid was centrifuged at 1000 rpm for 10 min and the supernatant was stored at −80 °C until further experiments.

Uterine endometrial tissues were obtained by biopsy on the contralateral side to the most recent ovulation using a biopsy device (Fujihira Industry), as previously described (Badrakh et al., 2020). Endometrial tissue biopsy samples for gene expression analysis using next generation sequencer were frozen in TRIzol reagent (Thermo Fisher Scientific, MS, USA) and stored below −80 °C until further analysis.

Prior to sampling the endometrial tissues and uterine fluid, the endometrial cells were collected using cytology brush (Fujihira Industry), applied to a microscopic slide and subjected to Giemsa staining. The percentage of polymorphonuclear leukocytes in all samples were less than 5.0 %, indicating no subclinical endometritis in both normal and RBC groups.

In a field study, uterine fluid was collected from Holstein cows housed on 4 commercial dairy farms. The uterine horns ipsilateral to the ovary with ovulation were flushed individually on day 1 of the estrous cycle (normal group: $n = 5$, average of sampling was 119.2 ± 19.1 days after parturition; RBC group: $n = 8$, average of sampling was 332.4 ± 35.8 days after parturition). The collected flushing fluid was centrifuged at 1000 rpm for 10 min and the supernatant was stored at −20 °C until further experiments.

2.2. In vitro cell culture experiments

Bovine endometrial epithelial cells (BEEC) were purchased from Cell Applications (San Diego, CA, USA) and cultured in bovine endometrial cell basal medium (Cell Applications, B910-400) with growth supplement (B911-GS) in tissue culture dish (100 mm, SARSTEDT, Tokyo, Japan). BEECs were seeded at a density of 2×10^5 cells/well in 24-well culture plates (Thermo Fisher Scientific). On the next day, BEECs were treated with uterine fluid supernatant (10 % volume) collected from normal fertile cows and RBCs or LPS (O111:B4, 100 ng/ml, Sigma-Aldrich) for 24 h at 38 °C. Uterine fluid collected from five animals in each group was mixed and used for cell culture. In other study, BEECs were treated with or without nobiletin (20 or 40 μM, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) for 1 h, then further treated with uterine fluid supernatant collected from normal fertile cows and RBCs for 24 h at 38 °C. After incubation, supernatants were collected to analyze protein levels, and cells were collected using lysis buffer (PureLink RNA mini kit, Thermo Fisher Scientific) to analyze mRNA expression, and stored at −80 °C until further analysis. Data were obtained at least two independent runs ($n = 3$–4 in each treated group).

2.3. RNA extraction and RNA-seq analysis of uterine endometrial tissues and BEECs

Total RNA was extracted from bovine endometrial tissues using TRIzol reagent and from BEECs using a PureLink RNA Mini Kit (Thermo Fisher Scientific). The concentration and quality of RNA were examined using a DS-11 spectrophotometer (DeNovix Inc, DE, USA). RNA samples of uterine endometrial tissues were extracted from individual cows ($n = 5$ in each group), and RNA samples of BEECs were pooled in each group. RNA sequencing analysis was performed using BGI Japan (Hyogo, Japan). After assessing the RNA quality (RIN value ≥7.0) using a 2100 Bioanalyzer (Agilent Technologies, CA, USA), DNA nanoballs (DNB) SEQ standard mRNA sequencing libraries were prepared. The final ligation polymerase chain reaction (PCR) products were sequenced using the DNBseq platform (BGI Japan). After removing the reads mapped to rRNA and low-quality reads, clean reads were mapped onto the reference genome (Bos_taurus_UMD3.1.1) using hierarchical indexing for the spliced alignment of transcripts. Gene expression levels were calculated using the RSEM software package and differentially expressed genes (DEGs) between samples were identified using DESeq (fold change >2.00 and $P < 0.05$). Then, we carried out clustering analysis of DEGs and Kyoto Encyclopedia of Genes and Genomes (KEGG)

enrichment analysis for the signaling pathway annotation of DEGs.

2.4. Measurements of endotoxin and interleukin (IL)-8

Endotoxin concentrations, as an indicator of LPS, in the uterine fluid were determined using Limulus Color KY test (FUJIFILM Wako Pure Chemical Corporation) according to the manufacturer's instructions. The levels of IL-8 in BEEC supernatants were measured using a bovine ELISA kit (DIY1028B, Kingfisher MN, USA) according to the manufacturer's instructions. The absorbances at 405, 450, and 650 nm were measured using a microplate spectrophotometer.

2.5. Quantitative real-time PCR

Total RNA was extracted from BEECs as described above, and cDNA was produced using a commercial kit (ReverTra Ace; Toyobo, Osaka, Japan). Real-time quantitative PCR was performed using the CFX Connect™ Real Time PCR system (Bio-Rad, Hercules, CA, USA) and a commercial kit (Thunderbird SYBR qPCR Mix; Toyobo) to detect the mRNA expressions of *IL-8* or *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*). The primers used for real-time PCR were as follows: forward, 5′-cctcttgttcaatatgacttcca-3′ and reverse, 5′-ggcccactctcaataactctc-3′ for IL-8; forward, 5′- acagtcaaggcagagaacgg-3′ and reverse, 5′- ccacatactcagcaccagca-3′ for GAPDH. The expression levels of each target gene were normalized to the corresponding GAPDH threshold cycle (CT) values using the ΔΔ CT comparative method (Livak & Schmittgen, 2001).

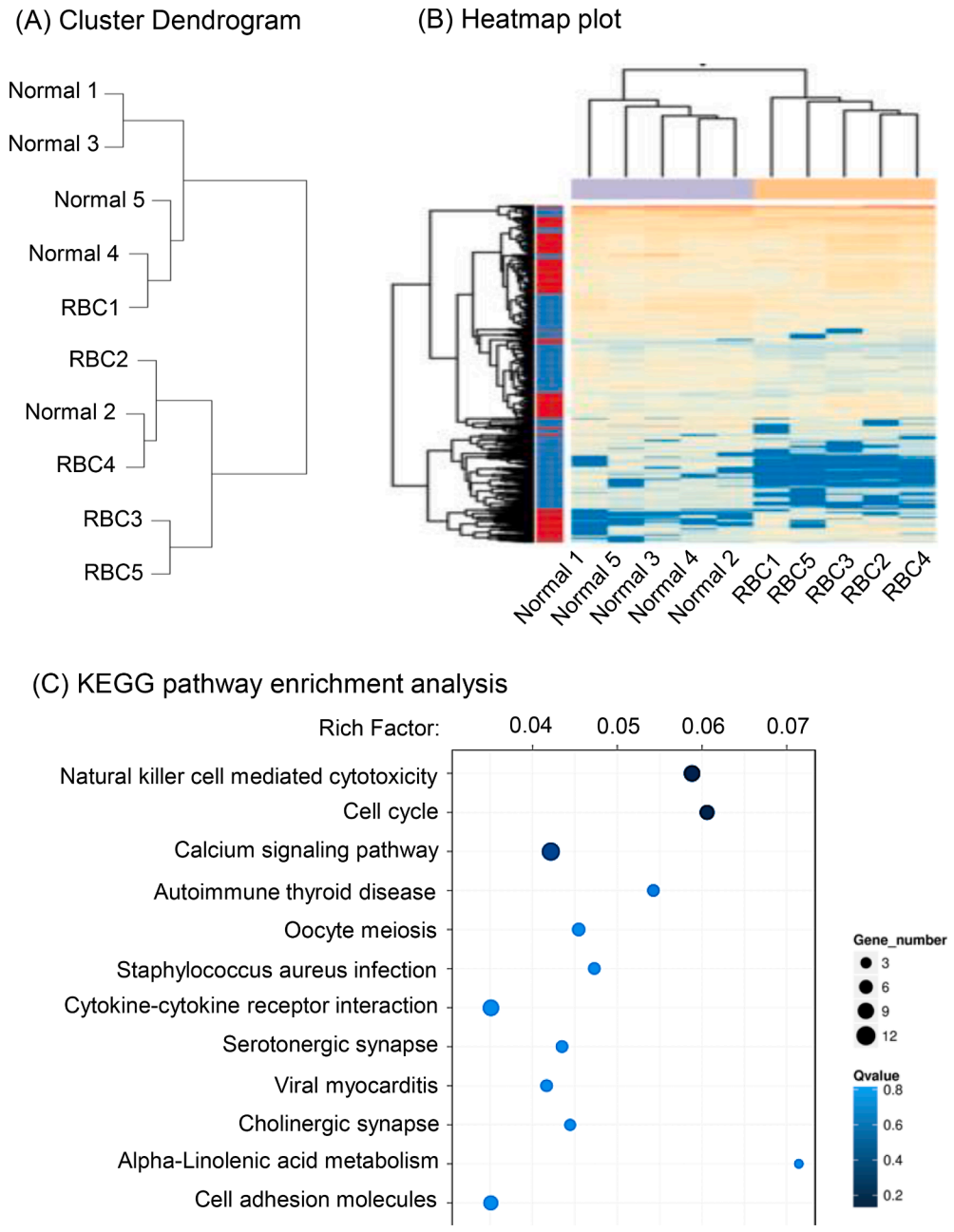

Fig. 1. Differentially expressed genes and pathways in uterine tissue from normal fertile cows and RBCs.
(A and B) The cluster dendrogram and heatmap plot analysis of differentially expressed genes in uterine tissues from normal fertile cows (normal) and RBCs. (C) KEGG enrichment analysis of differentially regulated between the normal and RBC groups. 12 pathways showed significant variations ($P < 0.05$).

2.6. Statistical analysis

Data are expressed as the mean ± standard error of the mean (SEM). Differences between treatment groups were identified using the Mann–Whitney *U* test. Multiple comparisons were made using one-way analysis of variance, followed by Tukey-Kramer multiple comparison tests using statistical software (Bell Curve, Social Survey Research Information, Tokyo, Japan). Statistical significance was set at $P < 0.05$.

3. Results

3.1. Differentially expressed genes and pathways in uterine tissue from normal fertile cows and RBCs

First, we performed cluster dendrogram and heatmap plot analysis of differentially expressed genes in uterine tissues from normal fertile cows (normal) and RBCs (Fig. 1A and B). In the cluster dendrogram analysis, only one individual (normal 2) in the control group was classified into a different cluster from the others (normal 1, 3, 4, and 5); however, there was a tendency for the clusters to separate into two groups (Fig. 1A). In the heatmap plot analysis, differences between the two groups (normal and RBC) were observed (Fig. 1B).

Gene profiles of uterine tissues from normal and RBC groups were analyzed, and 78 and 104 differentially expressed functionally known genes, respectively, were identified (*P*-value <0.05, log2 fold-change ≥1.0 as up-regulated genes or ≤ −1.0 as down-regulated genes). We showed up- and down-regulated functionally known genes in the uterine tissue of RBC compared with the normal group in Table 1 (top seven up-regulated known genes at adjusted *P*-value <0.1, and top eight down-regulated known genes at adjusted *P*-value <0.05). Other up- and down-regulated functionally gene groups with large variations are shown in Supplementary Tables 1 and 2.

KEGG enrichment analysis was performed to appraise pathways in the uterine tissue that were differentially regulated between the normal and RBC groups. As shown in Fig. 1C, 12 pathways showed significant variations ($P < 0.05$). In addition, we showed the top three pathways and their related genes (up and down-regulated genes) that changed in the uterine tissue of RBC compared with normal fertile group in Table 2. Natural killer (NK) cell mediated cytotoxicity was the top-ranked pathway, which included five up-regulated genes (Ig heavy chain Mem5-like [IgM5-like], interferon beta [IFNB], endogenous retrovirus group K member 7 [ERVK7] Env polyprotein-like, SH2 domain-containing protein 1A [SH2D1A], and granzyme B [GZMB]) and five down-regulated genes (integrin subunit alpha L [ITGAL], tyrosine-protein phosphatase non-receptor type 11 [PTPN11], UL16 binding protein 3 [ULBP3], putative killer cell immunoglobulin-like receptor like protein [KIR3DP1], UL16-binding protein 11 [UL16-BP11]). Cell cycle was second-ranked pathway, which included nine up-regulated genes (cyclin B2 [CCNB2], cell division cycle 25 C [CDC25C], budding uninhibited by benzimidazoles 1 [BUB1 mitotic checkpoint serine/threonine kinase B], pituitary tumor-transforming 1 [PTTG1], mitotic arrest deficient-like 1 [MAD2L1], TTK protein kinase [TTK], cyclin A2 [CCNA2], DNA replication licensing factor MCM4-like [MCM4-like], extra spindle pole bodies like 1, separase [ESPL1]). Calcium signaling pathway was the third-ranked pathway, which included two up-regulated genes (IgM5-like, tachykinin receptor 3 [TACR3]) and ten down-regulated genes (histamine receptor H2 [HRH2], T-cell receptor beta-1 chain C region [TRBC1], calcium channel, voltage-dependent, T type, alpha 1G subunit [CACNA1G], calcium channel, voltage-dependent, T type, alpha 1A subunit [CACNA1A], outer dense fiber of sperm tails 3-like 2 [ODF3L2], cholinergic receptor muscarinic 2 [CHRM2], ATPase, Ca^{++} transporting, plasma membrane 2 [ATP2B2], solute carrier family 8 (sodium/calcium exchanger), member 3 [SLC8A3], calcium channel, voltage-dependent, L type, alpha 1C subunit [CACNA1C], phospholamban [PLN]).

Table 1
Up- or down-regulated known genes in uterine tissues of RBCs compared with normal fertile cows.

Up-regulated known genes in uterine tissues of RBCs compared with normal fertile cows ($n = 5$ in each group)

Gene	Control	RBC	log2 Fold	P adj
Cadherin 12	28	151	2.43	0.012
Complement C1q like 1	17	78	2.20	0.031
Cell division cycle 25C	17	52	1.61	0.064
Centrosomal protein 55kDa	46	156	1.76	0.066
Solute carrier family 24 member 2	12	48	2.03	0.079
E2F transcription factor 8	19	63	1.75	0.079
Tryptase-2-like	1,613	4,031	1.32	0.079

Down-regulated known genes in uterine tissues of RBCs compared with normal fertile cows (n =5 in each group)

Gene	Control	RBC	log2 Fold	P adj
Complement C4-A-like	4,679	1,409	-1.73	0.012
LIM domain only 3	79	19	-2.09	0.018
Complement C4-like	4,389	1,676	-1.39	0.023
Integrin subunit alpha L	732	359	-1.03	0.031
Leukocyte cell derived chemotaxin 1	205	99	-1.05	0.031
Collagen type XXVII alpha 1	4,042	1,960	-1.04	0.043
Kielin/chordin-like protein	825	293	-1.49	0.047
Cationic amino acid transporter 3-like	32.7	5.8	-2.50	0.047

Table 2
KEGG pathway changed in uterine tissue of RBCs compared with normal fertile cows ($n = 5$ in each group).

Pathway 1: Natural killer cell mediated cytotoxicity	
Up genes	IgM5-like, IFNB, ERVK-7 Env polyprotein-like, SH2D1A, GZMB
Down genes	ITGAL, PTPN11, ULBP3, KIR3DP1, UL16-BP11
Pathway 2: Cell cycle	
Up genes	CCNB2, CDC25C, BUB1, PTTG1, MAD2L1, TTK, CCNA2, MCM4-like, ESPL1
Down genes	–
Pathway 3: Calcium signaling pathway	
Up genes	IgM5-like, TACR3
Down genes	HRH2, TRBC1, CACNA1G, CACNA1A, ODF3L2, CHRM2, ATP2B2, SLC8A3, CACNA1C, PLN

3.2. Differentially expressed genes and pathways after treatment with uterine fluid collected from normal fertile cows and RBCs

Since above results suggested that the uterine tissue conditions differ between normal fertile cows and RBCs, we thought that the condition of the uterine tissue was reflected in the uterine fluid, and examined whether the effect of uterine fluid on endometrial epithelial cells differed between the groups. The cluster dendrogram and heatmap plot analysis of differentially expressed genes in endometrial epithelial cells treated with uterine fluid from normal fertile cows and RBCs (Supplementary Fig. 1A and B). In both analyses, there were clear differences between the normal and RBC groups, indicating that the effect of uterine fluid on endometrial epithelial cells differed between the normal and RBC groups.

To appraise the pathways in the effect of uterine fluid that were differentially regulated between the normal and RBC groups, KEGG enrichment analysis was performed. As shown in Table 3, four or six pathways were changed by uterine fluid treatment with normal or RBC, respectively, that showed significant variation (*P*-value <0.01 and false discovery rate [FDR] <0.05).

In uterine fluid of the normal group, Wnt signaling pathway was the top-ranked pathway, which included eight genes (secreted frizzled-

Table 3
Pathways changed by treatment with uterine fluid of normal fertile cows or RBCs.

Top 4 pathways changed by treatment with uterine fluid of normal fertile cows

Rank	Pathway Name	P value	FDR	Changed known genes
1	Wnt signaling pathway	0.0000009	0.00017	SFRP4, WNT2, RAC2, MAPK10, WNT9A, RNF43, DAAM2, PORCN
2	Pathways in cancer	0.0002709	0.02628	STAT4, WNT2, RAC2, MAPK10, FGFR3, WNT9A, DAPK1, IL-15, BCL2
3	Mineral absorption	0.0007453	0.03679	ATP1A4, ATP1A2, SLC34A3, MT1A, S100G
4	Metabolic pathways	0.0007585	0.03679	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 12-like, UGT1A1, GLS2, A4GALT, ADH6, DGKQ, PDE7B, PTGES, ENTPD2, PLCH2, ACACB, COX7A1, SLC27A5, GPLD1

Top 6 pathways changed by treatment with uterine fluid of RBCs

Rank	Pathway Name	P value	FDR	Changed known genes
1	Metabolic pathways	0.0000003	0.00005	B3GALT1, MAN1C1, CDA, PKLR, DGKB, PIK3CG, GLS2, ST8SIA1, MGAT3, ADH6, DGKQ, LIPT2, PLCH2, AGMAT, SDS, ALOX12, AOC3, MGAT5B, QPRT, TM7SF2, GSTP1
2	Phospholipase D signaling pathway	0.0002272	0.02113	DGKB, PIK3CG, IL-8, DGKQ, SYK, LPAR6
3	Viral protein interaction with cytokine and cytokine receptor	0.0003963	0.02457	IL-8, GRO1, IL10RA, CCL2
4	Pathways in cancer	0.0006567	0.02681	GLI1, IL-8, FGFR3, WNT9A, LPAR6, MMP1, DLL3, FRAT2, GSTP1
5	NF-kappa B signaling pathway	0.0007208	0.02681	VCAM1, VCAM1-like, IL-8, GRO1, SYK
6	Malaria	0.0014450	0.04480	VCAM1, VCAM1-like, IL-8, CCL2

related protein 4 [SFRP4], wingless-type MMTV integration site family member 2 [WNT2], ras-related C3 botulinum toxin substrate 2 [RAC2], mitogen-activated protein kinase 10 [MAPK10], WNT9A, ring finger protein 43 [RNF43], dishevelled associated activator of morphogenesis 2 [DAAM2], and porcupine homolog [PORCN]). Pathways in cancer were the second-ranked pathway, which included nine genes (signal transducer and activator of transcription 4 [STAT4], WNT2, RAC2, MAPK10, fibroblast growth factor receptor 3 [FGFR3], WNT9A, death-associated protein kinase 1 [DAPK1], interleukin-15 [IL-15], and B-cell CLL/lymphoma 2 [BCL2]). Mineral absorption was the third ranked pathway, which included five genes (ATPase, Na^+/K^+ transporting, alpha 2 polypeptide [ATP1A4], ATP1A2, solute carrier family 34 [type II sodium/phosphate cotransporter], member 3 [SLC34A3], metall othionein-1A [MT1A], and S100 calcium binding protein G [S100G]). Metabolic pathway was the fourth ranked pathway, which included 14 genes (NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 12-like, UDP glucuronosyltransferase 1 family, polypeptide A1 [UGT1A1], glutaminase 2 [GLS2], alpha 1,4-galactosyltransferase [A4GALT], alcohol dehydrogenase 6 [ADH6], diacylglycerol kinase theta [DGKQ], phosphodiesterase 7B [PDE7B], prostaglandin E synthase [PTGES], ectonucleoside triphosphate diphosphohydrolase 2 [ENTPD2], phospholipase C eta 2 [PLCH2], acetyl-CoA carboxylase beta [ACACB], cytochrome c oxidase subunit VIIa polypeptide 1 [COX7A1], SLC27A5, and glycosylphosphatidylinositol specific phospholipase D1 [GPLD1]).

In uterine fluid of the RBC group, metabolic pathway was the top-ranked pathway, which included 21 genes (beta-1,3-galactosyl-transferase 1 [B3GALT1], mannosidase alpha class 1C member 1 [MAN1C1], cytidine deaminase [CDA], pyruvate kinase, liver and RBC [PKLR], diacylglycerol kinase beta [DGKB], phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit gamma [PIK3CG], GLS2, ST8 alpha-*N*-acetyl-neuraminide alpha-2,8-sialyltransferase 1 [ST8SIA1], mannosyl (beta-1,4-)-glycoprotein beta-1,4-N-acetylglucosaminyltrans ferase [MGAT3], ADH6, DGKQ, lipoyl(octanoyl) transferase 2 [LIPT2], phospholipase C eta 2 [PLCH2], agmatinase [AGMAT], serine dehydratase [SDS], arachidonate 12-lipoxygenase [ALOX12], amine oxidase, copper containing 3 [AOC3], MGAT5B, quinolinate phosphoribosyl-transferase [QPRT], transmembrane 7 superfamily member 2 [TM7SF2], glutathione S-transferase pi 1 [GSTP1]). Phospholipase D signaling pathway was the second-ranked pathway, which included six genes (DGKB, PIK3CG, chemokine (C-X-C motif) ligand 8 [CXCL8, IL-8], DGKQ, spleen tyrosine kinase [SYK], lysophosphatidic acid receptor 6 [LPAR6]). Viral protein interaction with cytokine and cytokine receptor was the third-ranked pathway, which included four genes (IL-8, chemokine (C-X-C motif) ligand 1 [GRO1], interleukin 10 receptor subunit alpha [IL10RA], chemokine (C-C motif) ligand 2 [CCL2]). Pathways in cancer was the fourth-ranked pathway, which included nine genes (GLI family zinc finger 1 [GLI1], IL-8, FGFR3, WNT9A, LPAR6, matrix metallopeptidase 1 [MMP1], delta-like 3 [DLL3], frequently rearranged in advanced T-cell lymphomas 2 [FRAT2], GSTP1). NF-kappa B signaling pathway was the fifth-ranked pathway, which included five genes (vascular cell adhesion molecule 1 [VCAM1], VCAM1-like, IL-8, GRO1, SYK). Malaria was the sixth-ranked pathway, which included four genes (VCAM1, VCAM1-like, IL-8, CCL2).

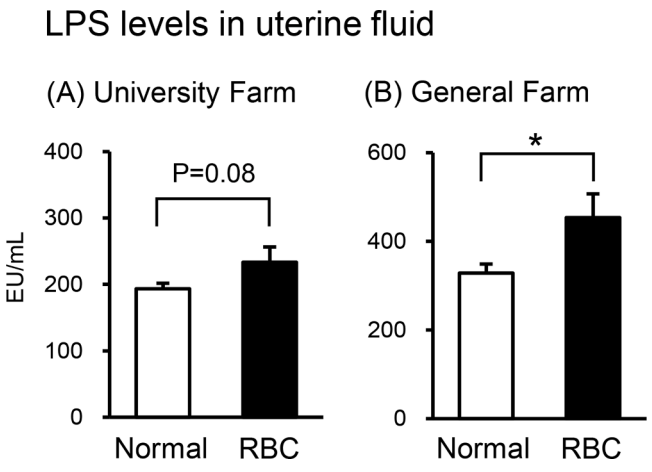

Fig. 2. LPS levels in uterine fluid from normal cows and RBCs.
(A and B) LPS levels in uterine fluid collected from normal fertile cows and RBCs were detected both in university farm ($n = 5$ in normal group, $n = 5$ in RBC group) and general commercial farm ($n = 5$ in normal group, $n = 8$ in RBC group). The results are shown as mean ± SEM. $^*P < 0.05$.

3.3. LPS levels in uterine fluid and the effect of LPS or uterine fluid from normal cows and RBCs on bovine endometrial epithelial cells

As shown in Fig. 2A, LPS levels in uterine fluid collected from RBCs tended to be higher than in those from normal cows of university farms. As the breeding environment at university farms is comparatively constant, similar investigations were conducted using uterine fluid obtained from multiple general farmers, and LPS levels were significantly higher in the uterine fluid of RBCs than in normal cows (Fig. 2B).

Subsequently, we examined the effect of LPS as containing factor within uterine fluids on bovine endometrial epithelial cells. We considered IL-8 as an indicator of LPS response because LPS generally

stimulates inflammatory cytokines (Turner et al., 2014), including IL-8, and IL-8 was listed as response gene in the KEGG analysis of uterine fluid of RBCs (Table 3). As expected, treatment with LPS significantly stimulated IL-8 secretion and mRNA expression in endometrial epithelial cells (Fig. 3A and B).

Next, we examined the effects of uterine fluid collected from normal cows and RBCs on bovine endometrial epithelial cells. Although treatment with uterine fluid from normal cows slightly increased (but not significantly) IL-8 secretion, treatment with uterine fluid from RBCs significantly increased IL-8 secretion in endometrial epithelial cells (Fig. 3C). On the other hand, treatment with uterine fluid from RBCs, but not from normal cows, tended to increase IL-8 mRNA expression in endometrial epithelial cells (Fig. 3D).

3.4. Effect of nobiletin with uterine fluid from normal cows and RBCs on bovine endometrial epithelial cells

As shown in Fig. 4, in bovine endometrial epithelial cells, treatment with nobiletin suppressed IL-8 secretion and mRNA expression caused by uterine fluid from normal cows or RBCs.

4. Discussion

In the present study, we first performed a comprehensive gene expression analysis to identify differences in the uterus between normal fertile cows and RBCs. Compared with the uterine tissue of normal fertile cows, pathway of "NK cell mediated cytotoxicity" was picked up as the one that fluctuated the most in the uterine tissues of RBCs. It is well known that uterine NK cells, such as decidual NK cells and uterine NK cells, play an essential role to establish pregnancy (Mahajan et al., 2022). On the other hand, circulating $CD56^{dim}$ NK cells shows high cytotoxicity (Fukui et al., 2015). Moreover, the number of uterine NK cells is increased in the pre-pregnancy endometrium in conjunction with recurrent pregnancy failure compared to normal fertile women (Mahajan et al., 2022; Matteo et al., 2007). In addition, we showed that the uterine fluid of RBCs clearly regulated inflammation-associated pathways and molecules in endometrial epithelial cells. These findings suggest that inflammatory responses, such as NK cell system, are excessive in the uterus of RBCs, and it is expected that conceiving will be difficult to become pregnant.

The second pathway that was identified as altered in uterine tissues of RBCs was "cell cycle". Among these, CDC25C was identified as the molecule that increased the most in the uterine tissues of RBCs. CDC25C is a conserved protein that is important for regulating cell division, directing the dephosphorylation of cyclin B-bound CDC2, and triggering entry into mitosis (Sutaji et al., 2022). CDC25C is essential for the successful progression of endometrial decidualization during the implantation window (Wang et al., 2010). Sutaji et al. (2022) reported that five candidate genes, including CDC25C, were identified as dysregulated molecules in the endometrium of polycystic ovary syndrome by systematic review and bioinformatics analysis. In bovine subclinical endometritis, commonly observed in RBCs, cell cycle-related genes were differentially regulated in endometrial tissues (Hoelker et al., 2012). These findings suggest that abnormal cell cycle regulation of endometrial cells in RBCs is one of the causes of infertility, and that improving this abnormal uterine environment may contribute to successful fertility.

Compared to normal fertile cows, cadherin 12 (CDH12) and complement C4-A-like were identified as the most increased and decreased genes, respectively, in the uterus of RBCs. CDH12 is an adhesion molecules and member of the neural cadherin (N-cadherin) family (Golawski et al., 2022). Golawski et al. (2022) reported that CDH12 levels in peritoneal fluid were significantly higher in patients with infertility than in fertile women, suggesting that CDH12 may play a role in the pathogenesis of infertility. On the other hand, the complement system plays a role in pregnancy, because several molecules involved in activation of complement system were up-regulated in pregnant animals (Walker et al., 2010). However, uncontrolled complement activation induces placental alterations resulting in adverse pregnancy outcomes including early pregnancy loss, pre-term birth and preeclampsia (Chighizola et al.,

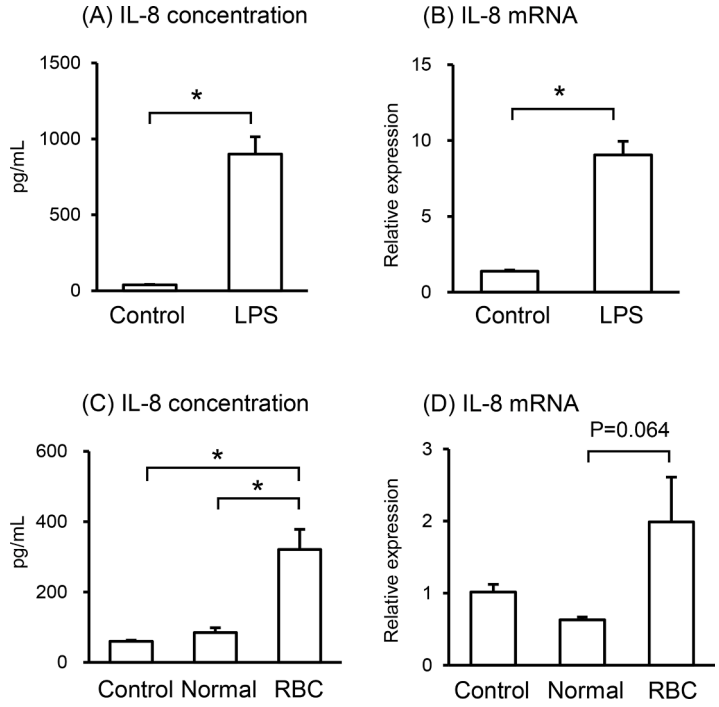

Fig. 3. The effect of LPS or uterine fluid from normal cows and RBCs on bovine endometrial epithelial cells.
(A and B) Bovine endometrial epithelial cells were treated with or without LPS, subsequently IL-8 secretion and mRNA expression were determined. (C and D) Bovine endometrial epithelial cells were treated with or without uterine fluid from normal fertile cows and RBCs, subsequently IL-8 secretion and mRNA expression were determined. Data are representative of two independent runs ($n = 3$–4). The results are shown as mean ± SEM. *$P < 0.05$.

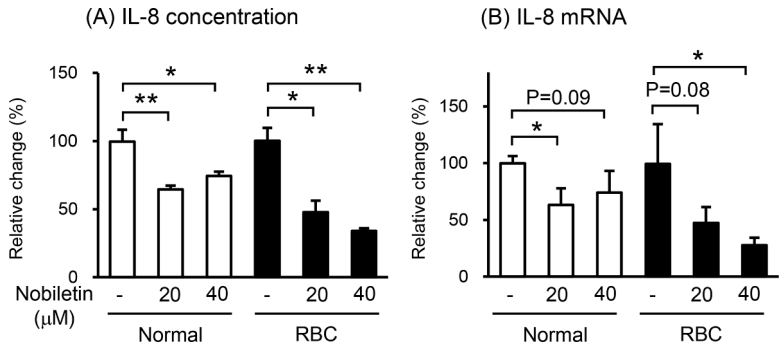

Fig. 4. Effect of nobiletin with uterine fluid from normal cows and RBCs on bovine endometrial epithelial cells.
(A and B) Bovine endometrial epithelial cells were pre-treated with or without nobiletin, subsequently further treated with uterine fluid from normal fertile cows and RBCs. Then, IL-8 secretion and mRNA expression were determined. Data are representative of two independent runs ($n = 3$–4). The results are shown as mean ± SEM. $^{*}P < 0.05$ or $^{**}P < 0.01$.

2020).

Uterine fluid plays an important role in reproductive success and is a complex mixture of molecules secreted primarily by endometrial epithelial cells and immune cells (Kasvandik et al., 2020; Lee et al., 2015). Components of uterine fluid are affected by maternal conditions; for example, the levels of IL-1β and MMP activity in the uterine fluid of patients with recurrent implantation failure were significantly higher than those in the control women (Inagaki et al., 2003). Because of the difference in gene expression in the uterine tissue between normal cows and RBCs in the present study, we hypothesized that the effects of uterine fluid on endometrial epithelial cells may also differ between normal fertile cows and RBCs. As expected, many inflammation-related pathways and genes were identified in the RBC uterine fluid-treated group. The uterine fluid of normal fertile cows regulated the "Wnt signaling pathway" as the top-ranked pathway in endometrial epithelial cells. Wnt/β-catenin signaling is a well-known key system for female reproduction, as either depletion or overexpression of Wnt/β-catenin in the endometrium leads to implantation failure in mice (Lai et al., 2023; Mohamed et al., 2005). In addition, the genes in Wnt/β-catenin signaling was significantly down-regulated in patients with recurrent implantation failure (Lai et al., 2023). These findings suggest that the appropriate regulation of Wnt signaling is essential for controlling the uterine environment for pregnancy. On the other hand, the uterine fluid of the RBCs regulated "Metabolic pathways" as top-ranked pathway in endometrial epithelial cells and cytidine deaminase (CDA) was included as down-regulated gene in this pathway. Evans et al. (2020) investigated proteins associated with human endometrial epithelial receptivity and identified key "epithelial receptome" 5 proteins, including CDA, as potential marker of "fertility determinants". Therefore, we suggest that the down-regulation of CDA by the uterine fluid of RBCs contributes to the low receptivity of bovine endometrial cells and infertility.

Postpartum uterine inflammation reportedly occurs in up to 40 % of dairy cows and is associated with infertility with a bacterial infection of the uterine cavity, such as *Escherichia coli* (*E. coli*) (Sheldon et al., 2009, 2006). It has been reported that 52.7 % of RBCs have subclinical endometritis; therefore, uterine infections, such as LPS, are important risk factors for a lesser reproductive performance of dairy cows (Heidari et al., 2019; Salasel et al., 2010). Since the inflammatory effects of *E. coli* are mainly induced by the endotoxin LPS, we determined the LPS levels in uterine fluid in the present study. Importantly, LPS levels in the uterine fluid were higher in RBC group than in the normal group. Since it is known that bacterial flora exists in the uterus of cows (Yagisawa et al., 2023), it is possible that LPS is produced from them and exists in the uterus. Additionally, the uterine fluid of RBCs clearly up-regulated inflammation-related pathways and molecules such as IL-8, GRO1, and CCL2, as shown in Table 3, suggesting that uterine fluid, including LPS, directly induces inflammatory responses in endometrial epithelial cells. In support of a previous report (Ding et al., 2020; Turner et al., 2014), treatment with LPS and uterine fluid of RBCs stimulated IL-8 mRNA expression and secretion in endometrial epithelial cells. Magata et al. (2022) reported that intrauterine LPS administration induces uterine inflammation with ovarian dysfunction in rats. These findings suggest that inflammatory uterine conditions are associated with endometrial dysfunction, resulting in RBC syndrome.

We focused on nobiletin, a polymethoxylated flavonoid found in citrus fruit peel, because of its important anti-inflammatory, antioxidant, tumor suppressive roles (Ashrafizadeh et al., 2020). Indeed, administration of nobiletin reduced the levels of inflammatory cytokines and improved endometriosis in a mouse model (Wei & Shao, 2018). We showed that treatment with nobiletin clearly suppressed IL-8 mRNA expression and secretion stimulated by uterine fluid both in normal and RBC. These findings suggest that nobiletin played a potential role in inhibiting chronic uterine inflammation. In addition to its beneficial effects on the uterus, nobiletin enhances the development and quality of bovine embryos (Canon-Beltran et al., 2021). Moreover, Zhao et al. (2023) reported that dietary feeding with citrus flavonoid extracts, including nobiletin, resulted in increased milk production, decreased systemic inflammation and endotoxin levels, and altered fecal microbiota in dairy cows. These results indicated the beneficial role of nobiletin in the reproductive function of cows. In future studies, it will be important to clarify the effect of nobiletin on RBS. Moreover, we suggest that searching for substances that can effectively improve uterine inflammation in RBC, including nobiletin, and it is necessary to develop methods for improving the inflammatory state of the uterine environment in RBCs.

5. Conclusion

From our data that the detailed differential gene expression of the uterus and the responses to uterine luminal fluid between RBCs and normal fertile dairy cows, the uterine environment of RBCs was found to be in inflammatory condition, resulting in a lower reproductive performance. Moreover, nobiletin may be able to suppress inflammatory-related cytokine production in the uterus.

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Ethical approval and consent to participate

All the experimental procedures complied with the Guidelines for the Care and Use of Agricultural Animals of Tokyo University of Agriculture

and all the animal protocols were approved by our institutional animal experiment committee.

CRediT authorship contribution statement

Maho Taru: Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Taiga Katoh:** Investigation, Formal analysis, Data curation, Conceptualization. **Karen Koshimizu:** Investigation, Formal analysis, Data curation. **Sohei Kuribayashi:** Investigation, Formal analysis, Data curation. **Ryotaro Miura:** Writing – review & editing, Formal analysis, Data curation, Conceptualization. **Seizo Hamano:** Writing – review & editing. **Koumei Shirasuna:** Writing – original draft, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.vas.2024.100369.

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