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Research Article

The Effects of Presynch-10 and Ovsynch on Some Endometrial Toll- and Nod-like Receptor Gene Expressions in Repeat Breeder Cows [1] [2]

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Abstract

Subclinical endometritis (SE) is one cause of repeat breeder syndrome (RBS). When endometrial cells come into contact with antigens during inflammatory events like endometritis, they act like innate immune system cells. Ovsynch-based therapies stimulate the innate immune system, which is how they may be able to cure SE and then RBS. This study aimed to assess the effects of presynch-10 and ovsynch applications on mRNA expressions of some endometrial Toll- and Nod-like receptors (TLRs and NLRs) in cows with RBS. A total of 40 repeat breeder cows were used in the study. The presynch-10 and ovsynch protocols were used in group 1 (G1, n=20), and no protocol was used in group 2 (G2, n=20). Endometrial samples were collected before and after the synchronization protocols in G1. In G2, samples were collected, and after waiting time up to the duration of synchronization protocol in G1, samples were taken again. The expression profiles of the genes being investigated were examined using RT-qPCR. NLRC4, NALP3 and TLR4 were basically expressed in all of the samples while NOD2 expression was completely undetectable. There was a statistical difference when NOD1 and TLR2 expressions in the second samples were compared between G1 and G2 ($P<0.05$). It was concluded that presynch-10 and ovsynch administration in cows may have a positively effect on the innate immune response and so be effective in the treatment of SE.

Keywords: *Endometritis, Cow, Innate immunity, Pattern recognition receptors*

Repeat Breeder İneklerde Presynch-10 ve Ovsynch Uygulamalarının Bazı Endometriyal Toll ve Nod Benzeri Reseptör Genlerinin Ekspresyonları Üzerine Etkileri

Öz

Subklinik endometritis (SE), repeat breeder sendromunun (RBS) nedenlerinden biridir. Endometriyal hücreler, endometritis gibi yangısal olaylar sırasında antijenlerle karşılaştıklarında, doğal bağışıklık sistemi hücreleri gibi davranırlar. Ovsynch temelli tedaviler doğal bağışıklık sistemini uyararak SE ve sonra RBS'yi tedavi edebilmektedirler. Bu çalışma presynch-10 ve ovsynch uygulamalarının RBS'li ineklerde bazı endometrial Toll- ve Nod- benzeri reseptörlerin (TLR'ler ve NLR'ler) mRNA ekspresyonları üzerindeki etkilerini değerlendirmeyi amaçlamıştır. Çalışmada toplam 40 repeat breeder inek kullanılmıştır. Grup 1'de (G1, n=20), presynch-10 ve ovsynch uygulanmış, Grup 2'de herhangi bir uygulama yapılmamıştır (G2, n=20). G1'de senkronizasyon uygulamaları öncesi ve sonrasında endometriyal örnekler alınmıştır. G2'de örnekler alındıktan sonra G1'de uygulanan senkronizasyon protokolü süresi kadar beklenerek örneklemeler tekrarlanmıştır. İncelenen genlerin ekpresyon düzeyleri RT-qPCR ile belirlenmiştir. NLRC4, NALP3 ve TLR4'ün temel olarak tüm örneklerde eksprese edildiği, NOD2 ekspresyonunun ise belirlenemeyecek seviyelerde kaldığı saptanmıştır. G1 ve G2'den alınan ikinci endometriyal örnekler arasında NOD1 ve TLR2 ekspresyonları arasında istatistiksel bir fark oluşmuştur ($P<0.05$). İneklerdeki presynch-10 ve ovsynch uygulamalarının doğal bağışıklık yanıtını olumlu yönde etkileyebileceği ve SE tedavisinde etkili olabileceği sonucuna varılmıştır.

Anahtar sözcükler: *Endometritis, İnek, Doğal bağışıklık, Kalıp tanıyan reseptörler*

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INTRODUCTION

Repeat breeding syndrome (RBS) is one of the most important causes of infertility in dairy herds and prolongs the calving interval, which results in economic losses [1]. Repeat breeding syndrome is defined as cows younger than 10 years of age that fail to conceive after 3 consecutive inseminations while exhibiting normal estrus every 18 to 24 days, have no clinical disorders or pathological discharge in the genital organs [1,2]. The most common causes of RBS are subclinical endometritis (SE) [3], poor estrus detection [4], delayed increase in LH that delayed a rise in progesterone level [5], embryonic losses [6], environmental factors [7] and malnutrition [8]. When the microorganisms that play a role in the etiology of SE, which one of the most important causes of RBS, reach the uterus, they come into contact with the endometrium, which is the frontline of the genital tract defense. Endometrial cells have pattern recognition receptors (PRRs) which recognize pathogen-associated molecular patterns (PAMPs) on the surface of microorganisms. This allows them to function like a member of the innate immune system [9]. Studies have shown that PRRs are expressed in endometrial cells in humans [10] and many animal species [11]. TLRs and NLRs are important members of the PRR family. TLR2 is very important receptor involved in the recognition of the cell surface components of Gram-positive bacteria [12]. The interaction of TLR2 and its ligand triggers an inflammatory response. Similar to TLR2, activation of TLR4 also leads to an inflammatory response. TLR4 is responsible for recognizing Gram-negative bacteria, such as *Escherichia coli* [9]. NLRs are also known as the second line in the natural defense system after TLRs. Responses to microorganisms were generated by NLRs when TLR response was inadequate or microorganisms escaped TLRs [13]. NLRs recognize endotoxin, protein or RNA of many microorganisms [14-18], viral agents and fungi [19].

This interaction acts as a signal that initiates inflammatory reactions [20]. Luteal function deficiencies are another important factor that causes RBS. In this context, bacteria found in the uterus or inflammation in the endometrium may inhibit the release of luteinizing hormone (LH) and adversely affect follicular development, ovarian functions and the ovulation mechanism [21]. Therefore, one approach to resolving RBS related to ovarian dysfunctions or sub-clinical endometrial inflammation is administering ovulation synchronization protocols. In this way, the compromised ovulation mechanism can be made functional by hormonal intervention. Studies revealed that if the first injection of gonadotropin-releasing hormone (GnRH) is given when the diameter of the dominant follicle is 10 mm, the ovsynch protocol will be more successful [22]. Therefore, the success of ovsynch can be improved by administering two doses of prostaglandin F2-alpha (PGF2α) at an interval of 14 days before starting the ovsynch protocol. As a result, the dominant follicle can be brought to the proper size. It is further hypothesized that PGF2α administration is beneficial in the treatment of SE as it induces local immunity of uterus [23,24].

This study aimed to assess the effects of administering presynch-10 and ovsynch to change mRNA expressions of some TLRs and NLRs in the endometrial tissue of cows with RBS. Cytological and bacteriological samples were also investigated.

MATERIAL and METHODS

All procedures were approved by the Local Ethical Committee of the Experimental Research on Animals (No: 2013/14). Cows were housed in a free stall barn from November 2013 through November 2014 and fed *ad libitum* total mixed ration diet and fresh water.

Cows

Forty multiparous Holstein-Friesian cows with RBS were enrolled in this study. Cows with RBS were identified by reviewing dairy farm records and gynecological examinations. For this purpose, multiparous cows that had been inseminated three consecutive times but did not get pregnant were selected. Rectal, vaginal and ultra-sonographic examinations were performed to determine whether or not there were any pathological conditions in uterus or ovarium. Body condition scores (BCS) were determined using a 5-point scale (1= thin to 5= obese) [25]. The BCS's of the cows in the present study were between 3.25 and 3.5 and, were clinically healthy and there was a corpus luteum (CL) in the ovaries.

Study Design

Cytological, bacteriological and biopsy samples were collected from the endometrium for microbiological identification, polymorph nuclear leucocyte rates and detection of gene expressions from all cows. Blood samples were also collected from the tail vein to identify the blood levels of estradiol and progesterone. The cows were randomly divided into two groups.

In group 1 (G1, n=20), rectal, vaginal and ultrasonographic examinations were performed and then endometrial samples were collected 10 days before synchronization began. Then presynch-10 and ovsynch protocols [26] were administered. Blood samples were collected from the tail vein on injection days. Endometrial samplings and clinical examinations were repeated 10 days after the last injection of ovsynch, and blood samples were collected *(Table 1)*. The cows in group 2 (G2, n=20) were not given any medications. First, clinical examinations were performed, and endometrial and blood samples were taken. Following the waiting period as long as the synchronization protocol used in G1, estruses were followed-up. Corpora lutea were identified by rectal examination 10 days after estrus, after which clinical examinations were performed and all of the samples were collected again in G2.

Table 1. *Applications of synchronization, gynecological examination and endometrial sampling in G1*

Syncronization Protocols	Days	Applications
Presynch-10	-34	VI, RE, R-USG, ESS-1, EBS-1, ECS-1, BIS
	-24	1st Clo
	-10	2nd Clo
Ovsynch	0	1st Buse, BIS
	7	Clo
	9	2nd Buse, BIS
	10	Ovulation, BIS
	20	VI, RE, R-USG, ESS-1, EBS-1, ECS-1, BIS

VI: *vaginal inspection,* ***RE:*** *rectal examination,* ***R-USG:*** *rectal ultrasonography,* ***ESS:*** *endometrial swab sampling,* ***EBS:*** *endometrial biopsy sampling,* ***ECS:*** *endometrial cytology sampling,* ***BIS:*** *blood sampling,* ***Clo:*** *cloprostenol [estrumate, 500 µg, im],* ***Buse:*** *buserelin acetate [receptal, 10 µg, im]*

Bacteriologic and Cytologic Examination

Special swabs (Uterus Culture Swab with AMIES transport medium, Ref. 17214/2951, Minitube, Germany) and endometrial brushes (Cytobrush®, Ref.17214/2960, Minitube, Germany) were used for bacteriological and cytological sampling. The swab and cytobrush were inserted inside a stainless-steel catheter about 50 cm long to reach the uterine lumen without any contamination.

First, cytological samples were collected by rotating the cytobrush while in contact with the uterine wall. Cytological slides were prepared by rolling the cytobrush onto clean microscope glass. Slides were dried for 1-2 min in the air and then placed in special labeled transport containers. Samples were dyed with the May Grünwald Giemsa Method, and 200 cells were counted per slide. Bacteriologic samples were collected by swabs and, immediately placed in the AMIES medium and transported to the laboratory within 24 h. Samples were plated onto blood, Brucella, Campylobacter, SDA and MacConkey agars.

RNA Extraction and cDNA Synthesis

Endometrial biopsy samples were collected from the dorsal wall and intercaruncular area of the uterus with a bovine biopsy instrument (Kruuse, 63 cm, Kat no: 141700, Denmark). Collected samples were placed into microcentrifuge tubes, and snap-frozen in liquid nitrogen. Samples were transported to the laboratory in a nitrogen tank, transferred to a refrigerator (-86°C) and stored until analysis. Total RNA extracted from endometrial samples was performed using the Trizol method.

Purity and concentration evaluations were assessed with Thermo Scientific NanoDrop 2000®, and integrity was evaluated by electrophoresis in 0.8% agarose gel. DNase I (#EN0521) was used for DNA digestion, and the Thermo Scientific Revert Aid First Strand cDNA Synthesis Kit was used according to manufacturer recommendations for cDNA synthesis.

Real Time PCR

Primer sequences were designed using PrimerQuest and primer-BLAST (idt/PrimerQuest and ncbi/tools/primer-blast) software programs *(Table 2)*. Housekeeping genes (HKGs) (B2-GAPDH, Beta-Aktin, HPRT1, YHWAZ, SDHA, SUZ12, TUBA1, 18S) were examined in order to select those that were the most stable and suitable.

Hormone Analysis

Blood samples were collected on injection days and when the 1st and 2nd endometrial samples were collected. They were then centrifuged for three min at 3000 g to obtain blood serum. Serum samples were stored at -20°C until analysis. Serum estradiol and progesterone assays were performed using the ECLIA® method (Electrochemiluminescence Immunoassay) in an internationally certified laboratory (TURKAK, TS EN ISO/IEC 17025:2005, DUZEN Laboratory Group, Turkey).

Statistical Analysis

Normality tests were conducted for all of the data obtained from the study. The paired t test was used to compare cytological cell count data. The Chi-square test was used to compare microbiological data among groups. Statistical comparison of serum estradiol and progesterone levels were performed using the paired t test and Wilcoxon test, respectively.

Quantification of the RT-qPCR results was performed using the ROCHE Nano Lightcycler® software. The most stable and suitable gene was identified as YHWAZ and selected as a reference gene. Subsequently, this reference gene was used to normalize qPCR data. The normalization procedure was performed on the mathematical model of $2^{-\Delta\Delta Ct}$ reported by Livak and Schmittgen [29]. The Wilcoxon test was performed for data from the same cow, and the Mann-Whitney U test was performed to identify differences among the groups. Minitab 16.0® software was used for statistical analysis and box plot graphics.

RESULTS

The calving-to-first-service interval for cows in G1 and G2 was 85.95±19.79 and 90.05±21.77 respectively. The mean number of days in milk (DIM) was 205.6±55.60 in G1 and 236.25±54.51 in G2 (min: 150, max: 349). Since the mean DIM of the cows is 205.60±55.60 (min: 150, max: 349), >3% polymorphonuclear leukocyte (PMNL) was accepted as a threshold value for SE in the cytological evaluation [30].

There were 38 cows that had SE according to the first endometrial cytology samples (ECS1) taken from 40 cows, and this number constitutes 95% of all cows. On the

Table 2. *Primer sequences of genes used in this study*

Gene	Primer (5'-3') Forward, Reverse	PCR	Gen Accession Number	Reference
TLR2	5'-GGTTTTAAGGCAGAATCGTTTG-3' 5'-AAGGCACTGGGTTAAACTGTGT-3'	190bp	NM_174197	[27]
TLR4	5'-CTTGCGTACAGGTTGTTCCTAA-3' 5'CTGGGAAGCTGGAGAAGTTATG-3'	153bp	NM_174198	[27]
NLRC4 (Ipaf)	5'-CCAGGAAGTGCTGAGAAAGG-3' 5'-CCCTTTACGTTTGTGCCTGT-3'	214bp	NM_001192323.2	Designed using Primer3
NOD-1	5'GCTTATCCAGAACCAGATCAC-3' 5'CCTCTTCTCATCTTCAAAGACC-3'	142bp	NM_001256563.1	Design using idt/PrimerQuest and ncbi/tools/primer-blast
NOD-2	5'GTAGACTTCACTGAATCCCAAC-3' 5'GCTCTCCACACCCATAAATAC-3'	168bp	NM_001002889.1	Design using idt/PrimerQuest and ncbi/tools/primer-blast
NALP3	5'CAGAATCTCACCCACCTTTAC-3' 5'GTCTAACTCCAACACCTGAAG-3'	114bp	NM_001102219.1	Design using idt/PrimerQuest and ncbi/tools/primer-blast
YHWAZ	5'CTGAGCAAGGAGCTGAATTATC-3' 5'CTCTGTATTCTCGAGCCATCT-3'	162bp	NM_174814.2	Design using idt/PrimerQuest and ncbi/tools/primer-blast
B2-GAPDH	5'-GGCGTGAACCACGAGAAGTATAA-3' 5'-CCCTCCACGATGCCAAAGT-3'	119bp	NM_001034034.2	[28]
β-actin	5'-ATCGGCAATGAGCGGTTCC-3' 5'-GTGTTGGCGTAGAGGTCCTTG-3'	143bp	BT030480.1	[27]
HPRT1	5'GCTACTGTGTGCTTAGGAAAG-3' 5'CTACCGAAACCTACTGAAACAC-3'	114bp	NM_001034035.2	Design using idt/PrimerQuest and ncbi/tools/primer-blast
SDHA	5'- CGTTGTATGGAAGGTCTCTG-3' 5'- GATGGACCCGTTCTTCTATG-3'	126bp	NM_174178.2	Design using idt/PrimerQuest and ncbi/tools/primer-blast
SUZ12	5'-GAACACCTATCACACACATTCTTGT-3' 5'- TAGAGGCGGTTGTGTCCACT-3'	130bp	XM582605	Designed using Primer3
TUBA1	5'-GCCCTACAACTCCATCCTCA-3' 5'- ATGGCCTCATTGTCTACCA-3'	78bp	NM_001166505.1	Designed using Primer3
S18	5'-ATGCGGCGGCGTTATTCC-3' 5'-GCTATCAATCTGTCAATCCTGTCC-3'	204bp	NR_036642.1	Designed using Primer3

B2-GAPDH: *B2-glyceraldehyde-3-phosphate dehydrogenase,* ***β-actin:*** *beta actin,* ***HPRT1:*** *hypoxanthine phosphoribosyl transferase-1,* ***YHWAZ:*** *tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta,* ***SDHA:*** *succinate dehydrogenase complex, subunit A,* ***SUZ12:*** *suppressor of zeste 12,* ***TUBA1:*** *tubulin alpha 1a, S18: ribosomal protein S18, bp: base pairs*

Table 3. *Groups of cows according to PMNL and SE rates*

Groups	Percentage of PMNL (SE status)	ECS1		ECS2	
		n	%	n	%
G1	>0% - <3% (not SE)	0	0	1	5
	≥3% (SE)	20	100	19	95
G2	>0% - <3% (not SE)	2	10	0	0
	≥3% (SE)	18	90	20	100

PMNL: *polymorphonuclear leukocyte;* ***ECS:*** *endometrial cytology sample;* ***SE:*** *subclinical endometritis*

Table 4. *PMNL rates between groups*

Groups	PMNL (X±S)		
	ECS1	ECS2	P
G1	21.85±9.50	18.70±8.08	>0.05
G2	13.85±8.54	16.15±7.13	>0.05
P	>0.05	>0.05	

X: *mean;* ***S:*** *standard deviation;* ***P:*** *significance value;* ***ECS:*** *Endometrial cytology sample*

other hand, all of the cows in G1 had SE, and this number decreased to 19 after administering the presynch-10 + ovsynch protocol.

Eighteen cows had SE according to the evaluation of ECS1, and all cows had SE when the second endometrial cytology samples (ECS2) were evaluated in G2 *(Table 3)*. There was no statistical difference between the PMNL cell numbers in the ECS1 taken from G1 and G2 (P>0.05). Also, there were no statistical differences between the PMNL counts of ECS1 and ECS2 in both G1 and G2 (P>0.05) *(Table 4)*.

When the first endometrial swabs (ESS1) taken from all cows were evaluated, no pathogenic microorganisms were identified in 30 of 40 samples. According to the microbiological analyses, no pathogens were identified in 18 samples while *Citrobacter* sp. was identified in the other 2 samples in G1. In the second endometrial swabs (ESS2) taken after the synchronization protocol, there was no pathogen in 17 endometrial swabs while *Citrobacter* sp. and *E. coli* were isolated in the other 3 samples. In G2, no pathogenic agent was isolated in 12 of the ESS1 while *E. coli*, *Streptococcus* sp., *Candida* sp., *Klebsialla* sp. and *Aspergillus* sp. were isolated in the other samples.

***Table 5.** Isolated microorganism rates of G1 in and G2*

Isolated Microorganism	**G1 (n=20)**				**G2 (n=20)**			
	ESS1		**ESS2**		**ESS1**		**ESS2**	
	n	**%**	**n**	**%**	**n**	**%**	**n**	**%**
NPI	18	90	17	85	12	60	10	50
Citrobacter sp.	2	10	2	10	-	-	-	-
E. coli	-	-	1	5	4	20	7	35
CNS	-	-	-	-	-	-	1	5
Streptococcus sp.	-	-	-	-	1	5	1	5
Candida sp.	-	-	-	-	1	5	-	-
Klebsiella sp.	-	-	-	-	1	5	1	5
Aspergillus sp.	-	-	-	-	1	5	-	-

***NPI:** no pathogen was isolated; **CNS:** coagulase negative staphylococci; **ESS:** endometrial swab sample*

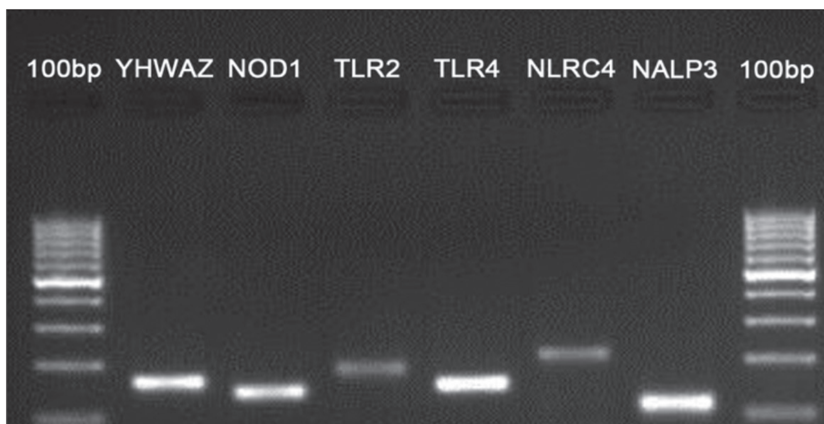

Fig 1. Agarose gel electrophoresis of RT-qPCR products from endometrial tissues. YHWAZ: tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide, NOD1: nod like receptor 1, TLR2: toll like receptor 2, TLR4: toll like receptor 4, NLRC4: NLR family CARD domain-containing protein 4, NALP3: NACHT-, LRR-, and pyrin domain-containing protein 3, bp: base pair

When ESS2 were examined, no pathogen was isolated in 10 samples. However, *E. coli, Streptococcus* sp., *Coagulase negative staphylococci* (CNS) and *Klebsiella* sp. Were isolated in the other 10 samples in G2 *(Table 5)*.

The resulting PCR products from YHWAZ, NOD1, NOD2, TLR2, TLR4, NLRC4 (Ipaf) and NALP3 were separated with electrophoresis on 2% agarose gel *(Fig. 1)*. The qPCR test results, melting curve analyzes and agarose gel images were evaluated together.

The geNorm®, NormFinder® and BestKeeper® programs were used to select the reference gene. YHWAZ was identified as the most suitable and stable reference gene. Expression levels of the genes being examined were determined based on statistical evaluations.

Data show that NLRC4, NALP3 and TLR4 were basically expressed in all samples while NOD2 expression was undetectable. There was a significant statistical difference when NOD1 expressions of EBS2s were compared between G1 and G2 ($P<0.05$). Similarly, there were differences in TLR2 expression between EBS1 and EBS2 in G2 ($P<0.05$) and between EBS2s in G1 and G2 ($P<0.05$) *(Fig. 2)*.

Serum progesterone levels were 8.52±3.83 ng/mL in G1 and 6.26±2.20 ng/mL in G2 in the first samples ($P>0.05$). In addition, progesterone and estradiol levels were identified in G1 on the days indicated in *Fig. 3* and *Fig. 4*. No statistical difference was observed between the estradiol levels while progesterone levels showed significant differences between the first (6.56±1.67) and second (3.20±0.03) samplings ($P<0.05$).

DISCUSSION

RBS is one of the most important problems affecting fertility, causing economic loss in dairy farming [31,32]. SE is one of the major etiologic factors of RBS in high milk-yielding cows, and it causes infertility, delayed onset of postpartum ovarian activity, prolongation of the luteal phase and a decline in the rate of conception [33]. The most commonly used technique to diagnosis SE is endometrial cytology using the cytobrush method [34]. Diagnosis is made by counting the PMNLs in the slides prepared from samples taken from the endometrium. But, the threshold number of cells required to diagnose SE is still a matter of debate. Various studies have been conducted on this subject, and different PMNL cells ratios have been accepted as a threshold based on DIM values [23,35,36]. Salasel et al.[30] defined a threshold of 3% PMNL for samples

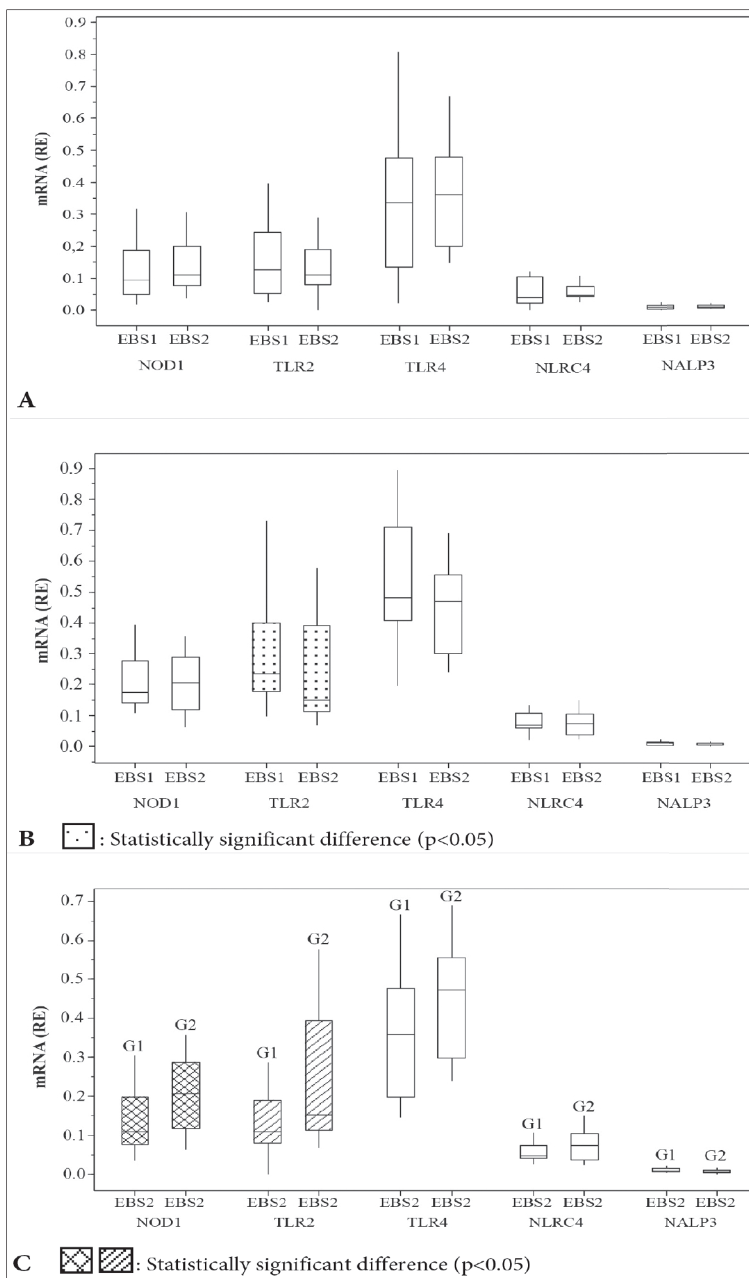

Fig 2. Box plot graphics of gene expressions. **A-** Transcription levels of NOD1, TLR2, TLR4, NLRC4 and NALP3 between EBS1 and EBS2 in G1, **B-** Transcription levels of NOD1, TLR2, TLR4, NLRC4 and NALP3 between EBS1 and EBS2 in G2, **C-** Transcription levels of NOD1, TLR2, TLR4, NLRC4 and NALP3 between EBS2 in G1 and EBS2 in G2

collected from cows at 190 DIM based on the results of the ROC analysis. The 3% PMNL threshold for diagnosis of SE was used in this study since the mean value of DIM was 205.6±55.6. Many studies have shown that the incidence of SE varies between 12-94% in the etiology of RBS [34,35]. The 95% SE rate calculated in this study is consistent with these studies.

One of the treatments of RBS caused by SE is the administration of presynch-10 and ovsynch. However, the use of PGF2α or GnRH in the treatment of endometritis on cytological recovery is controversial. For example, it has been reported in various studies that PGF2α stimulates the release of proinflammatory cytokines from the endometrium, induces local immunity and for these reasons can be used for SE treatment [37]. Nevertheless, there are also some studies which indicate that PGF2α administration has no statistically significant effect on the treatment of cytologic SE [23,38,39]. Similarly, cows with active CL cytologically diagnosed with SE were administered PGF2α for treatment, the cytological examination was performed again after treatment and there was no decrease in the prevalence of cytological SE [23]. In another study, GnRH administration was performed to stimulate ovulation in 128 cows at postpartum days 17±3. No significant differences were found between the control and the SE group based on the cytological examination on postpartum day 35±3 [39]. Histological studies have shown that leukocytes and neutrophils accumulate in the deep layers of the endometrium, such as stratum compactum [40]. Therefore, PMNL cells may not be found in the cytological samples taken from endometrium in

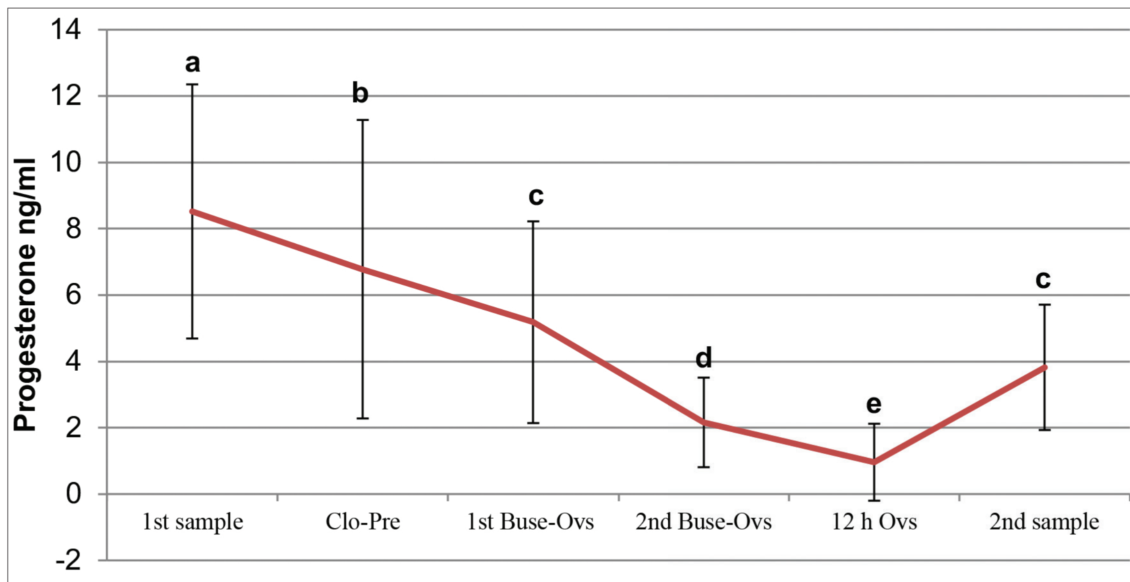

Fig 3. Progesterone levels by administration days in G1. a:b:c:d:e, P<0.05, **Clo-Pre:** 1st cloprostenol injection of presynch-10; **1st Buse-Ovs:** 1st buserelin acetate injection of ovsynch; **2nd Buse-Ovs:** 2nd buserelin acetate injection of ovsynch; **12 h Ovs:** 12 h after ovsynch

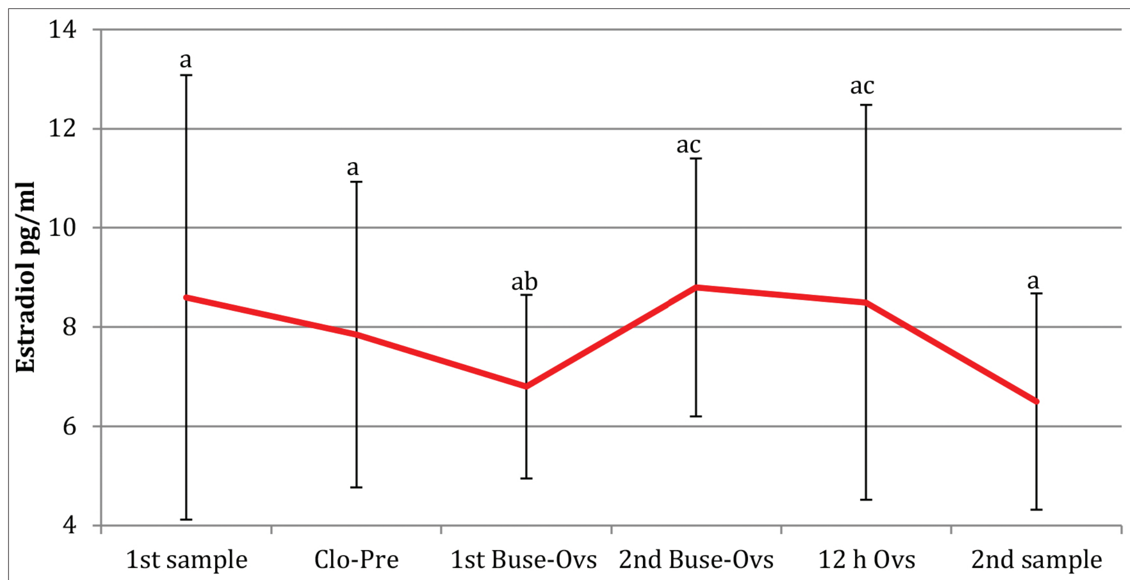

Fig 4. Estradiol levels by administration days in G1. b:c, P<0.05, **Clo-Pre:** 1st cloprostenol injection of presynch-10; **1st Buse-Ovs:** 1st buserelin acetate injection of ovsynch; **2nd Buse-Ovs:** 2nd buserelin acetate injection of ovsynch; **12 h Ovs:** 12 h after ovsynch

chronic SEs [41]. The reason that there is no cytological difference in the samples before and after presynch-10 and ovsynch administration in G1, or between the two samples in G2 may be that the cells are located in the deep layers of the endometrium. In addition to cytology, bacteriological examination is also important to evaluation of SE. Many different bacterial strains can be identified in clinical or subclinical endometritis in cows. For example, *Trueperella pyogenes* is the most prevalent microorganism in clinical endometritis [42] and is less common in SE [43]. In addition, it has been shown that the increase in the PMNL count is associated more with *T. pyogenes* than other pathogens [44]. *T. pyogenes* was not be identified in any microbiological cultures made from the 40 endometrial samples. However, *Coagulase negative staphylococci,* which is considered an opportunistic microorganism of the genital tract, and *E. coli*, *Bacillus* sp. and *Citrobacter* sp. which are classified as uterine pathogens in cows, and fungi, such as *Aspergillus* sp. were identified at various concentrations. The microbiological results indicate that there was no microbiological improvement in the samples whether taken before or after prescynch-10 and ovsynch. In this context, it can be assumed that the synchronization protocols have no microbiological healing effect in

cows with RBS. However, the statistical difference found between the first and second samples in G2 may be coincidental. The results of microbiological analyses have revealed that a wide variety of microorganisms are isolated in cows with RBS and SE. This situation suggests that endometrial microbiological findings are coincidental in RBS with SE [3]. Moreover, serum estradiol and progesterone levels were also monitored to determine whether the presynch-10 and ovsynch administrations were effective or not. And, as a result of the statistical analysis; hormone levels in the first serum samples of all cows in G1 and G2 were revealed to be consistent with progesterone and estradiol levels in the late diestrus of cows [45]. This also shows that they were in the same stage of the estrus cycle. In addition, the hormone levels in the other blood samples collected from G1 were compatible with the measured hormone levels with applications similar synchronization protocols in a different study [46]. On the other hand, progesterone levels measured during ovsynch were found to be different from our findings in another study. When the study examined, it was determined that as presynch, PGF2α was administered 2 days before ovsynch and the CLs were lysed [47]. Therefore, the measured progesterone levels were less than our findings. Because the last PGF2α injection was administered to 10 days before starting ovsynch. Namely, it was an active CL when ovsynch started. These results suggest that there was no abnormality in the ovarian functions of these cows and, administered synchronization protocols were effective.

After all these evaluations, the effects of synchronization methods administered on expression levels of some TLR and NLR genes were assessed. TLR2 and 4 are members of the TLR family that contribute to the innate immune response by stimulating the release of proinflammatory cytokines, and it has been determined that they were expressed in the endometrial cells of cows. In addition, the expression profiles of these receptors reportedly do not change according to the region where the samples are taken from in the uterus [27]. Recent studies revealed that TLR4 expression was higher in healthy and fertile cows than infertile ones with chronic endometritis at approximately 200 days postpartum [48]. Lower TLR4 expression in chronic endometritis has been associated with repeated or prolonged antigen stimulation [49]. There was no difference in TLR4 gene expression levels between or within groups, which is probably due to the fact that subclinical infections were chronic at that time. When the NLR expression profile was examined; it was concluded that the level of NLRC4 expressions did not change in all biopsy samples because the *Salmonella* could not be isolated from any endometrial swab. The activation of NLRC4 occurs when Salmonellar flagellin bind to NLRC4 [20]. The NOD2 and NALP3 are other members of NLRs and they are intracellular receptors. Muramyl dipeptide (MDP), which is a minimal structure of the bacteria's peptidoglycan cell wall, is a ligand that activates NOD2 and NALP3. MDP must be in cytosol to activate NOD2 and NALP3 [50]. NALP3 expression levels were low, and there were no differences between the samples. Similarly, NOD2 expression remained at undetectable levels. The low and undetectable expression levels of these genes may be due to the absence of activators in the cytosol. Likewise, this may be due to these receptors not being affected by prolonged inflammatory stimulus or synchronization protocols.

Tissue stress or malfunction could induce the innate immune response, which is referred to as para-inflammation. In this context, the reason for higher TLR2 and NOD1 expressions in EBS1 than EBS2 in G2, may be due to immune system stimulators, such as poor environmental conditions or malnutrition of the endometrial cells due to their malfunction and prolonged inflammation [51]. The increases seen in both TLR2 and NOD1 in the EBS2s of G1 supported the hypothesis that synchronization protocols may have immunomodulatory effects. These findings may be due to decreased progesterone level in EBS2 than EBS1 or administered PGF2α injections during presynch-10 and ovsynch. Because it is known that progesterone suppresses local immunity of uterus and, the synthesis of prostaglandins. PGF2α administered during synchronization caused luteolysis and, decreased progesterone concentration. Besides, PGF2α has also immunomodulatory effects independent of progesterone concentration [52]. However, these administrations did not affect the expression profiles of other receptor genes. These data showed that the mechanisms linking the steroids to immunity are yet to be fully elucidated and many studies need to be done in reproductive immunity.

Our data show that except for NOD2 all of the receptor genes examined were expressed in the endometrial cells of cows with SE. All of the other gene expressions were identified in all groups. These gene expressions could indicate that the mucosal innate immune system of endometrium plays various roles in RBS caused by SE. Furthermore, this study revealed that expressions of TLR-2 and NOD1 genes could be affected by ovulation synchronization methods related to GnRH and PGF2α.

Hence, a potential reason for the low success rate of implantation is subclinical endometritis in cows that may damage tissues. This damage could induce an inflammatory process by releasing some cytokines that are also stimulated by the activation of TLR and NLRs, thus initiating an inflammatory process and leading to embryo implantation failure, which is one of the causes of RBS. In this context, it was concluded that presynch-10 and ovsynch administration in cows positively affected the innate immune response and may be effective in the treatment of subclinical endometritis. Finally, the future potential scope of our research is to investigate the effects of TLR2 and/or NOD1 on the etiology of RBS.

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