

Effects of Subclinical Mastitis on Serum Estradiol and Tumour Necrosis Factor Alpha Levels During Estrus in Dairy Cows ^[1]

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Abstract

The effect of subclinical mastitis on serum estradiol and tumor necrosis factor alpha (TNF- α) levels during oestrus and subsequent fertility was investigated in dairy cows. 40 cows were divided into two groups as healthy control (n=20) and subclinical mastitis (n=20), according to the results of California Mastitis Test (CMT) and bacteriological isolation and identification. Cows were synchronised with a standart 7 day Ovsynch protocol. Following prostaglandin F2alpha (PGF2 α) administration, cows were examined with trans-rectal ultrasonography at 24, 36 and 48th h, dominant follicle diameters were recorded and blood samples were collected. Sixteen h after the second gonadotrophin-releasing hormone (GnRH) administration, cows were inseminated and a final examination of ovaries were performed and dominant follicle diameters were recorded. estradiol and TNF- α concentrations were analysed with ELISA in serum samples. No significant differences were found between the follicular diameters and growth patterns (P>0.05) of the two groups while estradiol concentrations were significantly higher in the subclinical mastitis group than the control group at 24 and 48 h after PGF2 α injection (P=0.017 and P=0.036 respectively). Also TNF- α levels were significantly higher in cows with subclinical mastitis than the control group (P=0.03). Positive correlations were observed between estradiol and TNF- α levels, in both groups (Control Group: R=0.512, P=0.021; Subclinical Mastitis Group: R=0.826, P<0.001). Overall pregnancy rate was higher in the control group (40%) than the subclinical mastitis (25%) group however the difference was not statistically significant (P>0.05). In conclusion estradiol and TNF- α concentrations were found higher in cows with subclinical mastitis during estrus and this data may be due to a luteal insufficiency during the initiation of synchronization, however further studies are required.

Keywords: Cow, Subclinical mastitis, Estradiol, TNF- α

Sütçü İneklerde Östrüs Sırasında Serum Östradiol ve Tümör Nekrozis Faktör Alfa Düzeyleri Üzerine Subklinik Mastitislerin Etkisi

Özet

Süt ineklerinde subklinik mastitislerin serum östradiol ve tümör nekrozis faktör alfa (TNF- α) düzeyleri ve fertilité üzerine etkileri araştırıldı. Kaliforniya Mastitis Test (CMT) ve bakteriyolojik izolasyon identifikasyon sonuçlarına göre 40 inek sağlıklı kontrol (n=20) ve subklinik mastitisli (n=20) olmak üzere iki gruba ayrıldı. İnekler standart bir 7 günlük Ovsynch protokolü ile senkronize edildi. Prostaglandin F2 alfa (PGF2 α) uygulamasını takiben inekler 24, 36 ve 48. saatlerde transrektal ultrasonografi ile muayene edildi, dominant follikül çapları kaydedildi ve kan örnekleri toplandı. İkinci gonadotropin salgılatıcı hormon (GnRH) uygulamasını takiben 16. saatte inekler tohumlandı, son bir ovaryum muayenesi yapıldı ve dominant follikül çapları kaydedildi. Serum örneklerinde östradiol ve TNF- α düzeyleri ELISA ile belirlendi. Folikül çapları ve folliküler gelişim özelliği yönünden iki grup arasında önemli bir fark görülmezken (P>0.05), serum östradiol konsantrasyonları subklinik mastitis grubunda kontrol grubuna göre PGF2 α enjeksiyonunu takiben 24 ve 48. saatlerde belirgin ölçüde yüksek bulundu (sırasıyla, P=0.017 ve P=0.036). Benzer şekilde TNF- α düzeyleri subklinik mastitisli ineklerde kontrol grubuna göre anlamlı düzeyde yüksekti (P=0.03). Her iki grupta da östradiol ve TNF- α düzeyleri arasında pozitif korelasyonlar bulundu (Kontrol Grubu: R=0.512, P=0.021; Subklinik Mastitis Grubu: R=0.826, P<0.001). Toplam gebelik oranı kontrol grubunda (%40) subklinik mastitisli gruba (%25) göre daha yüksek bulundu ancak bu fark istatistiksel açıdan anlamlı değildi (P>0.05). Sonuç olarak subklinik mastitisli ineklerde östrus sırasında östradiol ve TNF- α düzeylerinin yükseldiği, bunun senkronizasyonun başlangıcındaki bir luteal yetersizliğe bağlı olabileceği ve bu konuda daha fazla çalışmanın gerekli olduğu belirtildi.

Anahtar sözcükler: İnek, Subklinik mastitis, Östradiol, TNF- α



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INTRODUCTION

It has been well documented that both clinical and subclinical mastitis have negative impact on reproductive performance of dairy cows [1-4]. The interval for first postpartum insemination were delayed and the service for conception rate significantly increased in cows experiencing clinical mastitis compared to non-mastitic cows [2,4,5]. Long term nature of subclinical infections can damage the long process of follicular growth at various time points. It was observed that 32% of cows having subclinical mastitis exhibited low follicular estradiol concentrations compared to the uninfected and other subclinically infected cows. Low follicular estradiol levels in these cows were thought to be originated from the abnormally low expression of luteotrophic hormone (LH) receptor and steroidogenic genes in both theca and granulosa cell layers. However follicular estradiol concentrations in the remaining two-thirds of the subclinically infected cows were not affected and the reason of this difference remained unclear [6].

The release of proinflammatory cytokines such as interleukins, tumor necrosis factor- α (TNF- α) and eicosanoids, as a result of intramammary infections, have been suspected to be the possible causes of alterations in steroid production inside the follicular environment. The release of these substances in milk have been clearly observed during acute phase of challenge with experimental *Escherichia coli* Lipopolysaccharide (LPS) endotoxin [7]. Moreover in-vitro treatment by TNF- α reduced theca and granulosa cells androstenedione and estradiol production, respectively whilst the in-vivo effects were less pronounced [8]. Although these results have been observed clearly in clinical mastitis cases caused by Gram negative bacteria or via exposure to LPS endotoxin, mucopeptide layers of Gram positive pathogens also have been reported to possess the capability for inducing pyretic and cytokine responses like TNF- α [9].

Most of the previous studies have been focused on the effect of clinical mastitis on reproduction while the information about the subclinical cases remains relatively low. In two studies the increase in levels of milk TNF- α in naturally occurred subclinical mastitis cases have been reported [10,11]. Only in one of these systemic concentrations of TNF- α were measured and found to be unaffected [10]. In the present study our aim was to observe follicular growth patterns, estradiol and TNF- α concentrations in serum of healthy cows and cows with subclinical mastitis in order to detect a possible detrimental effect of subclinical mastitis on these parameters thus pregnancy rates obtained at a single fixed time artificial insemination.

MATERIAL and METHODS

The study was conducted in a dairy herd with a total size of 120 Holstein cows in the district of Izmir city. The cows were housed in tie-stall barns, connected with open air

pens year-round without pasturing. Cows had free access to water, mainly fed with corn silage and concentrate feed (Crude Protein: 16.72%, 2800 kcal/kg) and bedded on mats inside the housing. The cows were milked twice daily and average milk production in the course of study was 32 kgs. The study was conducted between November 2014 - May 2015. A total of 40 multiparous cows with an average of 145 days in milk, were included in the study during monthly visits as groups consisting of 8 cows approximately. Cows (mean age: 4.8 years) free from any kind of infectious disease, especially checked out for clinical mastitis, metritis and foot problems were chosen for the study. All procedures used in the study were approved by the Local Ethical Committee of Adnan Menderes University (ADÜ-HADYEK-Session number: 64583101/2014/059).

Primarily a standard Ovsynch protocol were administered starting by an injection of 2.5 mL of GnRH (Buserelin acetate, 0.004 mcg/mL, i.m. inj. sol., Receptal®, Intervet, Turkey) regardless of the stage of the cycle. On Day 7 a single dose of 5 ml PGF₂ α (Dinoprost tromethamine, 5 mg/mL i.m. inj. sol., Dinolytic®, Zoetis, Turkey) was injected. On 24th, 36th and 48th h after PGF₂ α injection, both ovaries were examined for dominant follicle by a portable real time B-mode ultrasonography (USG) equipped with a 5-7.5 MHz linear probe (Hasvet 838, Hasvet, Antalya) and the size of the dominant follicles were recorded. In addition to the examinations, each time a blood sample were collected into silicone coated tubes from *vena jugularis*. The blood samples were kept in room temperature for several h for clot formation and then sera were harvested following centrifugation with 3.000 rpm for 10 min. The serum samples were dispensed into microcentrifuge tubes, labeled and stored in -20°C until the time of hormone analysis. On 48th h upon transrectal ultrasonography and blood samplings the second GnRH injection were administered. Following this, at 16-18 h a final USG examination performed, the size of the follicle recorded and the cows were inseminated by the herd's veterinarian. Finally pregnancy diagnoses were performed by transrectal USG around day 45 following artificial insemination to all cows.

On the first day of examinations, in other words at the 24th h following the PGF₂ α administration in the milking parlor California Mastitis Test (CMT) was used to assign the cows into groups as healthy control (n=20) and subclinical mastitis (n=20). Cows having 2 or more mammary lobes CMT +1 or higher scores were included into the subclinical mastitis group. Following this milk samples from all cows were collected aseptically into tubes and these samples were taken immediately to the Laboratory of Department of Microbiology, Faculty of Veterinary Medicine, Adnan Menderes University (Aydin city).

Isolation and Identification of Pathogens

Milk samples were inoculated into blood agar and incubated at 37°C for 24 h. Samples obtained from the

colonies were than passed into brain heart infusion agar with 20% glycerine and stored in -20°C for further molecular analysis of bacteria type. At the end of the field study all of the stored samples were thawed and isolated bacterial DNA were extracted using Instagen DNA extraction kits (Instagene matrix, Bio-Rad, CN: 732-6030). Using PCR 16s rRNA, gene fragments were amplified and sequential analysis were done for bacterial identification. For this purpose S16S20 (5' AGA GTT TGA TCC TGG CTC AG 3') and 16S1390 (5' GAC GGG CGG TGT GTA CAA) universal primers were used. PCR products were visualised in 1% gel. These amplicons were sent to a commercial laboratory (Macrogen Inc./Korea) for sequential analysis. Later on the results of the sequential analysis were compared with the gene bank and species identification of the isolated bacteria were completed.

Analysis of Estradiol and TNF- α Levels

Stored serum samples were sent to a commercial laboratory (Farmasina, Istanbul/Turkey) for the analysis of estradiol and TNF- α using bovine species specific ELISA kits (Sunred Biotechnology Company®, China). The catalogue numbers, sensitivities and assay ranges and inter-assay and intra-assay coefficients of variations of these kits were as follows, respectively: Bovine estradiol ELISA kit (CN:21-04-210), 0.801 pg/mL, 1-300 pg/mL, <9%, <11%; Bovine TNF α ELISA kit (CN:201-04-0007), 14.155 ng/L, 15-4000 ng/L, <9%, <11%. While estradiol concentrations were measured in all of the samples, TNF- α were measured only in the first (24th h) samples.

Statistical Analysis

Statistical analyses were performed using the SPSS software version 22. For all variables tested, normality was checked by Shapiro-Wilk test. Intergroup relationships of the parameters (follicular size, estradiol, TNF- α) were analysed with Independent Samples T-test. The observed differences in parameters (follicular size, estradiol) by time were analysed with variance tests for repeated measures and Bonferroni pairwise comparisons. Correlations were analysed by Pearson correlation tests. Differences with P values less than 0.05 were considered to be significant.

The results were presented as the mean \pm SEM.

RESULTS

Intramammary Infections

The aseptically collected milk samples have revealed a variety of mastitis pathogens mostly consisting of Gram positive cocci from the 20 subclinically infected cows. All of the cows in the mastitis group had two or more mammary lobes infected and most of these cows had multiple pathogens residing in each mammary lobe. The types and distribution of these pathogens are demonstrated in *Table 1*.

Follicular Growth

Mean follicle diameters at 24, 36, 48 and 64th h were all slightly larger in the controls compared to the subclinically infected cows however these differences were not statistically significant ($P>0.05$). Also no statistically significant differences were observed in the follicular growth patterns throughout the repeated examinations inside each group ($P>0.05$). Follicle diameters throughout the study are presented in *Table 2*.

Table 1. The isolated and identified mastitis pathogens

Tablo 1. İzolasyonu ve identifikasyonu yapılan mastitis patojenleri

Isolated Patogens	No of Lobes Isolated	No of Cows Isolated
Gram Positive Pathogens		
<i>Staphylococcus aureus</i>	3	2
<i>Streptococcus agalactiae</i>	9	8
Coagulase negative Staphylococci (CNS)	13	9
<i>Corynebacterium spp.</i>	18	11
<i>Aerococcus viridans</i>	23	13
<i>Bacillus spp.</i>	7	4
Others	5	5
Gram Negative Pathogens		
<i>Escherichia coli</i>	4	3
Others	2	1

Table 2. Follicle diameters and estradiol concentrations at h after PGF₂ α administration. Results are expressed as mean \pm standard errors

Tablo 2. PGF₂ α uygulamasını izleyen saatlerde folikül çapları ve östradiol konsantrasyonları. sonuçlar ortalama \pm standart hata olarak ifade edilmiştir

Parameter	Group	Hours			
		24 th h	36 th h	48 th h	64 th h
Follicle Diameters (cm)	Control	1.85 \pm 0.06	2.01 \pm 0.07	2.1 \pm 0.07	2.13 \pm 0.07
	SubclinicalMastitis	1.79 \pm 0.05	1.99 \pm 0.06	2.05 \pm 0.05	2.07 \pm 0.05
P values		NS	NS	NS	NS
Estradiol (pg/mL)	Control	44.2 \pm 1.94 ^a	45.89 \pm 3.59	41.4 \pm 2.39 ^a	-
	SubclinicalMastitis	63.85 \pm 7.65 ^b	60.95 \pm 7.1	59.94 \pm 8.19 ^b	-
P values		P=0.017	NS	P=0.036	

Different superscripts in the same column (a,b) indicate significant differences, NS: Not significant ($P>0.05$)

Hormone Concentrations

Serum estradiol concentrations (pg/mL) of the subclinically infected cows were higher than the control group at 24 (63.85±7.65 and 44.2±1.94, respectively); 36 (60.95±7.1 and 45.89±3.59, respectively) and 48 (59.94±8.19 and 41.4±2.39, respectively) h while these differences were significant at 24 and 48th h (P=0.017 and P=0.036 respectively). The decrease in the mean estradiol concentration from 45.89±3.59 to 41.4±2.39 in the control group throughout 36 to 48th h were also significant (P=0.043).

TNF-α (ng/L) concentrations measured from the samples at 24th h after the PGF₂α injection in the subclinically infected and the control groups were 954.16±89.97 and 732.54±39.89 respectively and the difference was statistically significant (P=0.03).

Conception rate in the control group was higher than in the subclinical mastitis group and they were 40% (8/20) and 25% (5/20) respectively. However the difference was not found statistically significant (P>0.05).

Serum TNF-α was significantly correlated with estradiol concentrations at 24th h in both groups (Table 2). However no significant correlations were found between follicular size and estradiol concentrations (P>0.05).

DISCUSSION

PCR based bacterial identification methods like used in this study makes it possible to detect bacteria more sensitively at the species level [12]. The isolated pathogens were mostly consisted of *Aerococcus viridans*, *Corynebacterium spp.*, coagulase-negative staphylococci (CNS) and *S. agalactiae*. The first three bacteria most frequently isolated in this study, are known to reside on the cows teat skin, around ostium papillare and sometimes cause subclinical mastitis with mild rise in somatic cell counts [13]. *Aerococcus viridans* has been isolated from clinical and subclinical mastitis cases recently and the source of the bacteria reported as composted manure bedding [14,15]. Similar to this study *Aerococcus viridans* were isolated frequently from milk samples and cows could freely access to open pens where manure content was high. *Corynebacterium spp.* were not considered as causal agents of mastitis previously, however it was identified from cases of subclinical and clinical mastitis cases [12]. In this study also *Corynebacterium spp.* were isolated from 18 mammary quarters of 11 subclinically infected cows. CNS causes milder milk loss and lower somatic cell counts (SCC) compared to the major mastitis pathogens however they are reported to be an important cause of mastitis especially in well managed dairy farms [16,17]. The high frequency of the above bacteria isolated from quarters of subclinical mastitic cows and rather limited number of major mastitis pathogens such as *S. aureus*, *S. agalactiae* and *E. coli* indicates a similar

profile where the opportunistic bacteria leads to milder subclinical infections.

In this study follicular growth patterns were similar in both groups and no statistically significant differences were observed between groups at any time points (P>0.05). The mean diameters for control and mastitic groups at 24th h of PGF₂α injection were 1.85±0.6 and 1.79±0.5 respectively and in the proceeding examinations follicular sizes in all cows increased gradually. Compared to the previous studies concerning GnRH - PG protocol synchronized cows, our data shows larger ovulatory follicles [6,18,19]. Recently in a study multiparous cows which are subjected to the 7 day Ovsynch protocol demonstrated larger follicles than primiparous cows and the conception rate was not affected by follicle size but pregnancy loss between 32 to 60 days increased due to larger follicle sizes [20]. Likewise, in the present study all of the cows were multiparous and 7 day Ovsynch protocol were used which may explain the larger follicle diameters. Aside from this the follicular growth pattern in both groups were similar to each other and this was in accordance with the studies of Lavon et al. [6] and Lavon et al. [21].

Decreased steroid production capacity in the pre-ovulatory follicles of cows with either clinical or subclinical mastitis have been reported previously [6,21]. Follicular estradiol concentrations in one thirds of subclinically infected cows were significantly lower than healthy cows [6]. Also an extended estrus - ovulation interval accompanied with lower plasma estradiol concentrations were observed in 30% of cows with mastitis. However in this study serum estradiol concentrations were all higher in the subclinical mastitis group than the control cows and at 24th and 48th h and the differences were significant (P<0.05). Furman et al. [22] have reported that experimental subclinical intramammary infection did not cause an immediate decline in follicular estradiol but rather a marked, delayed decline in estradiol concentrations 16 days after mastitis induction was terminated. Moreover large portion of cows (especially consisting of Gram positive group) were not affected by the administration and follicular estradiol levels remained normal. These variations among cows were presumed to be related to individual, genetic differences. Authors have also suggested that the antral and medium sized follicles are more susceptible to stress and the effect of subclinical intramammary infections (IMI) thus occurs belatedly [22]. In the present study as previously discussed above the mean follicular diameters of cows were higher than observed in the other studies and consequently larger follicles may be less affected from subclinical mastitis.

Timed AI programs such as used in this study depends on the effect of an initial GnRH injection to ovulate or luteinize the existing follicles. The presence of a functional luteal structure has been shown to be an important factor in the development of the new follicular wave thus the preovulatory follicle. Progesterone supplementation via a

CIDR device to cows that do not have a functional luteal structure at the initiation of ovsynch protocol have significantly increased circulating progesterone levels and subsequent fertility^[23]. During early stages of diestrus progesterone concentrations are lower and LH pulses occur with greater frequency. Before the corpus luteum develops after ovulation, large estrogen-active follicles develop in the ovaries. As the luteal structure advances to mid phase, progesterone concentrations are increased and LH pulses are decreased. Administration of progesterone to ovariectomized cows also suppresses the release of LH, and both exogenous and endogenous sources has a similar suppressive action in inhibiting release of LH pulses from the anterior pituitary^[24]. TNF- α and IL-1 β release may cause endogen PGF₂ α release from the endometrium which in turn causes luteolysis^[25]. In the present study higher serum TNF- α levels in the subclinical mastitis group may have caused insufficient development of luteal structure causing lower progesterone levels at the time of first GnRH injection thus leading to greater release of LH pulses from the anterior pituitary causing higher estradiol levels. Higher LH pulses during the development phase of follicular wave may have caused estrogen active persistent follicles, which may not respond to GnRH treatment as indicated by Lopez-Gatius et al.^[26]. Consequently overall fertility may be decreased in the subclinical mastitis group due to low ovulation and/or fertilization rates, however the difference was not significant ($P > 0.05$).

No significant correlations between follicular diameters and estradiol levels at any time point in both groups were observed where this is a finding that could be expected in fixed time AI protocols especially in cows that did not exhibit estrus. Nearly 60% of cows did not display estrus behaviour during fix time AI protocols and follicular diameters were not correlated with serum estradiol concentrations in these cows^[27].

A series of studies have reported high milk and serum concentrations of TNF- α in natural or experimentally induced *E. coli* mastitis cases^[7,11,28,29]. Riollet et al.^[10] also have observed that in chronic *S. aureus* infections interleukin together with TNF- α , other cytokines such as IL-1 α , IL-1 β , IL-6, IL-10 and IL-12 regulatory cytokine mRNA were synthesized in cells derived from infected mammary glands however cell subpopulations in blood from infected cows were not modified, indicating that immune responses to chronic intramammary infections were not manifested systemically. However as TNF- α is an important marker in the modulation of immune responses to infections and pathogen - host related variations may occur during intramammary infections, researchers of the present study aimed to re-test the possible role of TNF- α in mastitis related follicular/steroidogenic alterations during estrus in cows. Our results demonstrated that at 24th h following PGF₂ α injection, mean serum TNF- α levels of subclinically infected cows were found significantly higher than the control cows ($P < 0.05$). This result is in contrast with the

findings of Riollet et al.^[7]. It can be said that in the present study multiplicity and variety of the pathogens may have caused this condition. However most of the pathogens were in Gram positive nature and a large portion of them were opportunistic mastitis bacteria causing mild to moderate rises in somatic cell counts. In addition serum TNF- α levels in both groups were at considerably low levels when compared to the researches demonstrating the TNF- α levels during endotoxin challenge^[7,30]. This seems to be in accordance with the mild nature of isolated pathogens that they may have caused much lower TNF- α release than endotoxemic bacteria but not totally absent.

Another interesting data in this study was that estradiol and TNF- α levels were significantly correlated in both sub-clinical mastitis group ($P < 0.001$, $R = 0.826$) and the control group ($P = 0.021$, $R = 0.512$). Kahl et al.^[30] have reported that the production of TNF- α during endotoxin challenge is enhanced during the follicular phase compared to the luteal phase in beef heifers. The above mentioned data in the present study may represent a similar effect of estradiol on the production of TNF α . Not presuming the effect of subclinical mastitis on TNF- α unsound, estradiol may have caused an amplifying effect on TNF- α levels. Sakumoto et al.^[31], have reported that in cell cultures obtained from theca and granulosa cells of bovine small (2-5 mm) and large (12-18 mm) follicles, exposure to TNF- α resulted in inhibition of estradiol secretion in small follicles, but did not in large follicles. The reason for this was the low TNF- α receptor expression in the granulosa and theca cells of preovulatory follicles. Concerning this data and regarding the follicular growth patterns and serum estradiol concentrations observed in this study, it can be said that preovulatory follicles were not affected by the TNF- α content in serum.

As this study was focused on follicular growth, estradiol and TNF α levels in cows during estrus, data concerning pre and post-synchronisation progesterone levels, luteal structures and ovulation response to the initial GnRH injection were lacking. Together with these and levels of other cytokines examined during diestrus and estrus more comprehensive data would be obtained.

In conclusion follicular growth patterns were not affected by subclinical mastitis while estradiol and TNF- α was higher in the cows with subclinical mastitis than in the control group. Estradiol and TNF- α concentrations were positively correlated and this may indicate a possible increasing effect of estradiol on TNF α . Authors have hypothesised that high estradiol levels in the cows with subclinical mastitis could be a result of luteal deficiency due to cytokine release, however this requires further studies with intense data.

REFERENCES

1. Moore DA, Cullor JS, Bondurant RH, Sischo WM: Preliminary field

- evidence for the association of clinical mastitis with altered interestrus intervals in dairy cattle. *Theriogenology*, 36, 225-236, 1991. DOI: 10.1016/0093-691X(91)90384-P
- 2. Barker AR, Shrick FN, Lewis MJ, Dowlen HH, Oilver SP:** Influence of clinical mastitis during early lactation reproductive performance of Jersey Cows. *J Dairy Sci*, 81, 1285-1290, 1998. DOI: 10.3168/S0022-0302(98)75690-5
- 3. Shrick FN, Hockett ME, Saxton AM, Lewis MJ, Dowlen HH, Oliver SP:** Influence of subclinical mastitis during early lactation on reproductive parameters. *J Dairy Sci*, 84, 1407-1412, 2001. DOI: 10.3168/jds.S0022-0302(01)70172-5
- 4. Santos JEP, Cerri RLA, Ballou MA, Higginbotham GE, Kirk JH:** Effects of timing of first clinical mastitis occurrence on lactational and reproductive performance of Holstein dairy cows. *Anim Reprod Sci*, 80, 31-45, 2004. DOI: 10.1016/S0378-4320(03)00133-7
- 5. Ahmadzadeh A, Frago F, Shafii B, Dalton JC, Price WJ, McGuire MA:** Effect of clinical mastitis and other diseases on reproductive performance of Holstein cows. *Anim Reprod Sci*, 112, 273-282, 2009. DOI: 10.1016/j.anireprosci.2008.04.024
- 6. Lavon Y, Leitner G, Klipper E, Moalle U, Meidan R, Wolfenson D:** Subclinical, chronic intramammary infection lowers steroid concentrations and gene expression in bovine preovulatory follicles. *Domest Anim Endocrinol*, 40, 98-109, 2011. DOI: 10.1016/j.domaniend.2010.09.004
- 7. Riollet C, Rainard P, Poutrel B:** Differential induction of complement fragment C5a and inflammatory cytokines during intramammary infections with *Escherichia coli* and *Staphylococcus aureus*. *Clin Diagn Lab Immunol*, 7, 161-167, 2000. DOI: 10.1128/CDLI.7.2.161-167.2000
- 8. Williams EJ, Sibley K, Miller AN, Lane EA, Jishwick J, Nash DM, Hearsh S, England GCW, Dobson H, Sheldon M:** The effect of *Escherichia coli* lipopolysaccharide and Tumor Necrosis Factor alpha on ovarian function. *Am J Reprod Immunol*, 60, 462-473, 2008. DOI: 10.1111/j.1600-0897.2008.00645.x
- 9. Huszenicza G, Janosi S, Gaspardy A, Kulcsar M:** Endocrine aspects in pathogenesis of mastitis in postpartum dairy cows. *Anim Reprod Sci*, 82-83, 389-400, 2004. DOI: 10.1016/j.anireprosci.2004.04.029
- 10. Riollet C, Rainard P, Poutrel B:** Cell subpopulations and cytokine expression in cow milk in response to chronic *Staphylococcus aureus* infection. *J Dairy Sci*, 84, 1077-1084. DOI: 10.3168/jds.S0022-32(01)745-7
- 11. Slebodzinski AB, Malinowski E, Lipczak W:** Concentrations of triiodothyronine (T3, tumour necrosis factor- α (TNF- α) and interleukin-6 (IL-6) in milk from healthy and naturally infected quarters of cows. *Res Vet Sci*, 72, 17-21, 2002. DOI: 10.1053/rvsc.2001.0514
- 12. Cantekin Z, Ergün Y, Doğruer G, Sarıbay MK, Solmaz H:** Comparison of PCR and culture methods for diagnosis of subclinical mastitis in dairy cattle. *Kafkas Univ Vet Fak Derg*, 21, 277-282, 2015. DOI: 10.975/kvfd.201.12309
- 13. Braem G, Vlieghe SD, Verbist B, Heyndrickx M, Leroy F, Vuyst L:** Culture-independent exploration of the teat apex microbiota of dairy cows reveals a wide bacterial species diversity. *Vet Microbiol*, 157, 383-390, 2012. DOI: 10.1016/j.vetmic.2011.12.031
- 14. Spakova T, Elecko J, Vasil M, Legath J, Pristas P, Javorsky P:** Limited genetic diversity of *Aerococcus viridans* strains isolated from clinical and subclinical cases of bovine mastitis in Slovakia. *Pol J Vet Sci*, 15, 329-335, 2012. DOI:10.2478/v10181-012-0051-1
- 15. Saishu N, Morimoto K, Yamasato H, Ozaki H, Murase T:** Characterization of *Aerococcus viridans* isolated from milk samples from cows with mastitis and manure samples. *J Vet Med Sci*, 77, 1037-1042, 2015. DOI: 10.1292/jvms.15-0100
- 16. Heinze J, Donat K, Brandt HR, Wehrend A:** Subclinical staphylococcal intramammary infections: Within-herd prevalence and effects on milk yield and somatic cell counts in Thuringian dairy herds. *Berl Munch Tierarztl Wochenschr*, 128, 61-69, 2015.
- 17. Vanderhaeghen W, Piepers S, Leroy F, Van Coillie E, Haesebrouck F, De Vlieghe S:** Identification, typing, ecology and epidemiology of coagulase negative staphylococci associated with ruminants. *Vet J*, 203, 44-51, 2015. DOI: 10.1016/j.tvjl.2014.11.001
- 18. Pursley JR, Mee MO, Wiltbank MC:** Synchronization of ovulation in dairy cows using PGF_{2 α} and GnRH. *Theriogenology*, 44, 915-923, 1995. DOI: 10.1016/0093-691X(95)00279-H
- 19. Peters MW, Pursley JR:** Timing of final GnRH of the Ovsynch protocol affects ovulatory follicle size, subsequent luteal function, and fertility in dairy cows. *Theriogenology*, 60, 1197-1204, 2003. DOI: 10.1016/S0093-691X(03)00120-1
- 20. Colazo MG, Behrouzi A, Ambrose DJ, Mapletoft RJ:** Diameter of the ovulatory follicle at timed artificial insemination as a predictor of pregnancy status in lactating dairy cows subjected to GnRH-based protocols. *Theriogenology*, 84, 377-383, 2015. DOI: 10.1016/j.theriogenology.2015.03.034
- 21. Lavon Y, Leitner G, Moallem U, Klipper E, Voet H, Jacoby S, Glick G, Meidan R, Wolfenson D:** Immediate and carryover effects of Gram-negative and Gram-positive toxin-induced mastitis on follicular function in dairy cows. *Theriogenology*, 76, 942-953, 2011. DOI: 10.1016/j.theriogenology.2011.05.001
- 22. Furman O, Leitner G, Roth Z, Lavon Y, Jacoby S, Wolfenson D:** Experimental model of toxin-induced subclinical mastitis and its effect on disruption of follicular function in cows. *Theriogenology*, 82, 1165-1172, 2014. DOI: 10.1016/j.theriogenology.2014.08.002
- 23. Bisinotto RS, Castro LO, Pansani MB, Narciso CD, Martinez N, Sinedino LD, Pinto TL, Van De Burgwal NS, Bosman HM, Surjus RS, Thatcher WW, Santos JE:** Progesterone supplementation to lactating cows without a corpus luteum at initiation of the Ovsynch protocol. *J Dairy Sci*, 98, 2515-2528, 2015. DOI: 10.3168/jds.2014-9058
- 24. Kinder JE, Kojima FN, Bergfeld EGM, Wehrman ME, Fike KE:** Progesterin and estrogen regulation of pulsatile LH release and development of persistent ovarian follicles in cattle. *J Anim Sci*, 74, 1424-1440, 1996.
- 25. Hansen PJ, Soto P, Natzke RP:** Mastitis and fertility in cattle - Possible involvement of inflammation or immune activation in embryonic mortality. *Am J Reprod Immunol*, 51, 294-301, 2004. DOI: 10.1111/j.1600-0897.2004.00160.x
- 26. López-Gatius F, Santolaria P, Yaniz J, Rutllant J, López-Béjar M:** Persistent ovarian follicles in dairy cows: A therapeutic approach. *Theriogenology*, 56, 649-659, 2001. DOI: 10.1016/S0093-691X(01)00596-9
- 27. Perry GA, Swanson OL, Larimore EL, Perry BI, Djira GD, Cushman RA:** Relationship of follicle size and concentrations of estradiol among cows exhibiting or not exhibiting estrus during fixed-time AI protocol. *Domest Anim Endocrinol*, 48, 15-20, 2014. DOI: 10.1016/j.domaniend.2014.02.001
- 28. Nakajima Y, Mikami O, Yoshioka M, Motoi Y, Ito T, Shikawa Y, Fuse M, Nakano K, Yasukawa K:** Elevated levels of tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6) activities in the sera and milk of cows with naturally occurring coliform mastitis. *Res Vet Sci*, 62, 297-298, 1997. DOI: 10.1016/S0034-5288(97)90209-5
- 29. Hoeben D, Burvenich C, Trevisi E, Bertoni G, Hamann J, Bruckmaier RM, Blum JW:** Role of endotoxin and TNF- α in the pathogenesis of experimentally induced coliform mastitis in periparturient cows. *J Dairy Res*, 67, 503-514, 2000.
- 30. Kahl S, Elsasser TH, Li CJ:** Variability in tumor necrosis factor- α , nitric oxide, and xanthine oxidase responses to endotoxin challenge in heifers: Effect of estrous cycle stage. *Domest Anim Endocrinol*, 36, 82-88, 2009. DOI: 10.1016/j.domaniend.2008.10.006
- 31. Sakumoto R1, Shibaya M, Okuda K:** Tumor necrosis factor- α (TNF α) inhibits progesterone and estradiol-17 β production from cultured granulosa cells: Presence of TNF α receptors in bovine granulosa and theca cells. *J Reprod Dev*, 49, 441-449, 2003. DOI: 10.1262/jrd.49.441