Postpartum subclinical endometritis, its diagnosis and impact on early reproductive parameters in dairy cows

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ABSTRACT

The present study was aimed at the diagnosis of subclinical endometritis and its impact on early reproductive parameters in postpartum dairy cows (N=45). The proportion of polymorphonuclear cells (PMNCs-%) and intraluminal uterine fluid (ILUF-mm) was assessed by cytotape and transrectal ultrasonography, respectively, for the diagnosis of subclinical endometritis at 8 weeks post-partum. A receiver operator characteristics curve was used to determine the diagnostic threshold of PMNCs and ILUF. The optimal threshold value for PMNCs was $\geq 6\%$ (area under the curve (AUC)-0.89; sensitivity-85.71%; specificity-83.33%; $P<0.05$) whereas it was ≥ 3.40 mm for ILUF (AUC-0.92; sensitivity-100%; specificity-83.33%; $P<0.05$). The diagnostic odds ratio (DOR) for the development of subclinical endometritis in cows having a PMNCs percentage and ILUF above the threshold level was 7.70 and 6.00, respectively. Early reproductive parameters, such as days needed for completion of uterine involution (UINV) and first postpartum ovulation (FPO) were studied and were reported to be significantly higher in cows diagnosed with subclinical endometritis based on the percentage of PMNCs ($P<0.01$; $P<0.05$ for UINV & FPO, respectively) and ILUF ($P<0.05$; $P<0.01$ for UINV & FPO, respectively). Linear correlation analysis showed a significant relationship ($p\leq 0.01$-0.05) between the proportion of PMNCs and early reproductive parameters. Similarly, a significant correlation ($P<0.01$) between ILUF and PMNCs was reported, irrespective of the threshold level. In conclusion, endometrial cytology and transrectal ultrasonography with a threshold level $\geq 6\%$ for PMNCs and ≥ 3.40 mm for ILUF, respectively, defined the presence of subclinical endometritis, and this led to a delay in the establishment of subsequent early post-partum reproductive performance.

Key words: diagnostic threshold; intraluminal uterine fluid; polymorphonuclear cells; reproductive parameters; subclinical endometritis

Introduction

The postpartum period is generally defined as the time between parturition and completion of uterine involution, and the partial resumption of ovarian cyclic activity, which usually occurs around 35 days later (SHELDON et al., 2004). Due to unavoidable contamination of the uterus during parturition, infections such as metritis and clinical/subclinical endometritis are the

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most common cause of decreased productivity and fertility in dairy cows (GILBERT, 2011). Subclinical endometritis is defined as the superficial inflammation of the endometrium, with no signs of systemic illness, characterized by an increase in the number of polymorphonuclear cells (PMNCs) inside the uterine lumen (KASIMANICKAM et al., 2004; PASCOTTINI and OPSOMER, 2016). For diagnostic purposes, endometrial cytology via cytotape, as a semi-invasive technique, is one of the most advanced and frequently employed methods for diagnosis of subclinical endometritis, based on the fact that the proportion of polymorphonuclear cells (PMNCs) increases during uterine inflammation (BARLUND et al., 2008; DUBUC et al., 2010). Transrectal ultrasonography, based on the level of intraluminal uterine fluid (ILUF), due to lower validation, has not been used extensively for the diagnosis of subclinical endometritis (SHARMA and SINGH, 2019). Therefore, the present study was carried out with two objectives: (i) to establish the diagnostic threshold of PMNCs and ILUF, and the impact of subclinical endometritis on early post-partum reproductive parameters; (ii) to establish inter-correlation and validate the use of a combination of semi- and non-invasive techniques in cows diagnosed with subclinical endometritis.

Materials and methods

Animals. Forty-five crossbred Jersey cows (N=45) with no ante- and post-partum complications (Parity 2-4), having been reared in a loose housing system under standard management conditions, fed a total mixed ration, once daily *ad libitum*, and with unrestricted access to water (32.6°N, 76.3°E, altitude 1290.8 m), were enrolled for the research. The cows did not receive any treatment during the prepartum period or the course of the study, and their health status was assessed on the basis of their body condition score (2.53±0.10, visual scoring system; EDMONSON et al., 1989), normal rectal temperature at calving (38.67±0.02ºC), and having had no difficulty in the expulsion of the fetus and placenta. The cows were milked twice daily (04:00 and 15:00 h). All the experiments were carried after receiving the approval of the ethical committee of the Dr. G.C. Negi College of Veterinary and Animal Sciences, CSKHPKV, Palampur.

Endometrial cytology. The cytotape method of endometrial cytology was employed for assessing the PMNCs percentage to diagnose subclinical endometritis at 8 weeks post-partum. The cytotape assembly was a modified version of cytobrush assembly (PASCOTTINI et al., 2015), and consisted of a stainless steel rod (an artificial insemination gun without the stylet), sterile paper tape (3M Micropore™), and a sanitary plastic sheath. The assembly was introduced into the vagina after cleaning the vulval area, and the sheath was perforated at the external os of the cervix, followed by the introduction of the steel rod rolled by a paper tape into the body of the uterus. The sample was taken by rolling the rod with the tape, on the wall of the uterine body, with gentle pressure of the index finger through the rectum. The cytotape was then retracted from the uterus and a smear was formed by gently rolling the tape on a clean glass slide. Prepared slides were air-dried, fixed in methanol for 15 minutes, and then stained with a modified Wright-Giemsa stain for 45 minutes. All the slides were evaluated by optical light microscope, and cells were counted in a total of 10 fields. The percentage of epithelial cells, endometrial cells and PMNCs (Figures 1a and 1b) were assessed at 40X magnification (RANA et al., 2020).

Transrectal ultrasonography for assessment of uterine fluid. The presence of intraluminal uterine fluid was determined by transrectal ultrasonography at 7.5 MHz frequency using a Mindray portable ultrasound device (Mindray Z5; VETMODEL 75L50EAV) at 8 weeks post-partum (Figures 2a and 2b). Measurements of uterine lumen (mm) were made in at least three locations and the point where the maximum amount of fluid was present was taken into consideration (DOUREY et al., 2011).
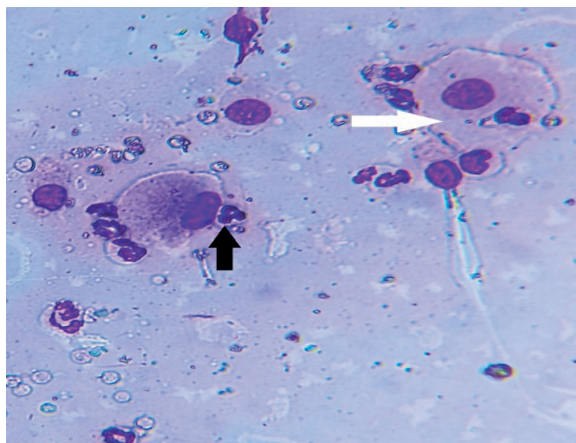
Fig. 1a. Endometrial cytology of uterus in dairy cows at 8 weeks post-partum diagnosed with cytological endometritis (Wright-Giemsa stain) - epithelial cell (white arrow) & PMNCs (black arrow) (40X)
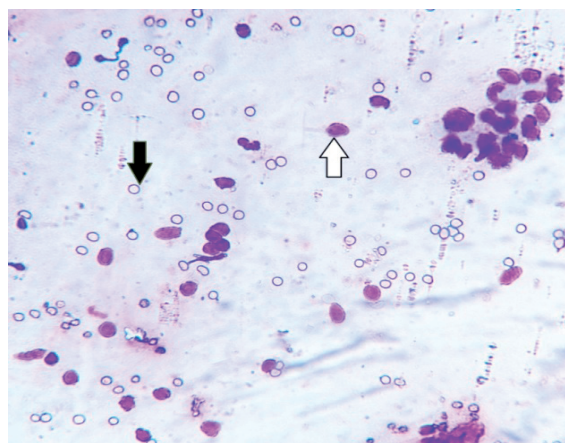

Fig. 1b. Endometrial cytology of uterus in dairy cows at 8 weeks post-partum not diagnosed with cytological endometritis (Wright-Giemsa stain) - endometrial cell (white arrow) & RBCs (black arrow) (40X)
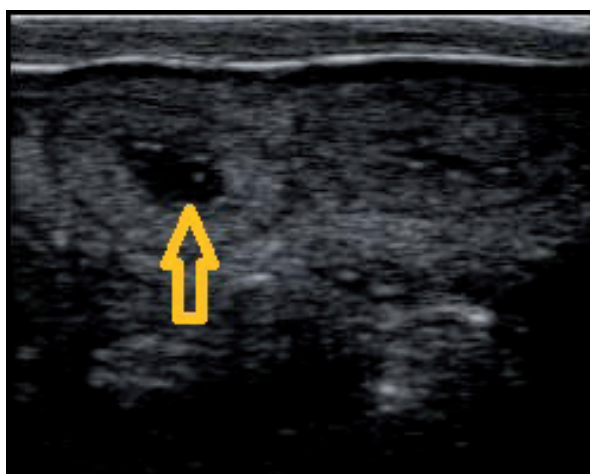

Fig. 2a. B-mode sonographic image of uterine horns in dairy cows at 8 weeks post-partum with ≥3.40 mm level of ILUF (yellow arrow)
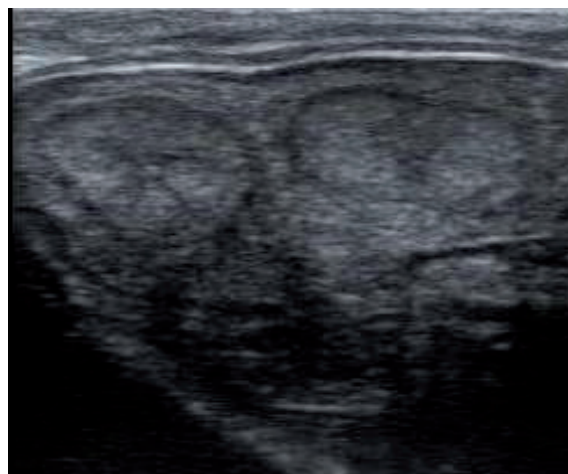

Fig. 2b. B-mode sonographic image of uterine horns in dairy cows at 8 weeks post-partum with <3.40 mm ILUF (white arrow)

Ultrasonographic monitoring of early reproductive parameters. Following parturition, the cows were monitored for completion of uterine involution (UINV) and first postpartum ovulation (FPO) at a weekly interval by transrectal ultrasonography. Uterine involution was considered complete when no further change took place between two consecutive examinations in the diameter of the uterine horns and both the previous gravid uterine horn (PGUH) and the previous non-gravid uterine horn (PNGUH) were nearly symmetrical i.e. a difference of ≤ 1mm (SHARMA et al., 2017). The first post-partum ovulation was characterized by the absence of a large anechoic structure (pre-ovulatory follicle), and the presence of small hypoechoic structure (*corpus luteum haemorrhagicum*) at the same site observed in the previous examination.

Statistical analysis. The data obtained were analyzed statistically using Student's t-test for

testing the significance of parameters and the receiver operator characteristics curve (ROC) to obtain the area under the curve, and to determine the threshold of the diagnostic parameters, and linear regression and correlation function using NCSS 2020, USA (Version 20.0.1).

Results

The receiver operator characteristics curve, shown in Figure 3, and the area under the curve (AUC) were determined to find the threshold levels of both the parameters for diagnosis of subclinical endometritis, i.e. ≥6% (AUC-0.89; P<0.05) and ≥3.40 mm (AUC-0.92; P<0.05) for PMNCs percentage and ILUF, respectively. The sensitivity and specificity of both the parameters at the threshold level is also mentioned in Figure 3. The diagnostic odds ratio (DOR) for the development of subclinical endometritis in cows with a PMNCs percentage and ILUF above threshold levels was 7.70 and 6.00, respectively (Figure 3). The overall percentage of PMNCs was also reported to be significantly higher (P<0.01) in cows diagnosed with PMNCs and ILUF above the threshold level.

AUC for PMNCs = 0.89 (p<0.05), 95% C.L.= 76.81-95.85
AUC for ILUF = 0.92 (p<0.05), 95% C.L.= 79.33-97.30
Cut–off level for PMNCs
PMNCs ≥ 6% (95% C.L.= 0.626-0.952), DOR= 7.70
Sensitivity = 85.71% and Specificity = 83.33%
Cut–off level for ILUF
ILUF ≥ 3.40 mm (95% C.L.= 0.626-0.952), DOR= 6.00
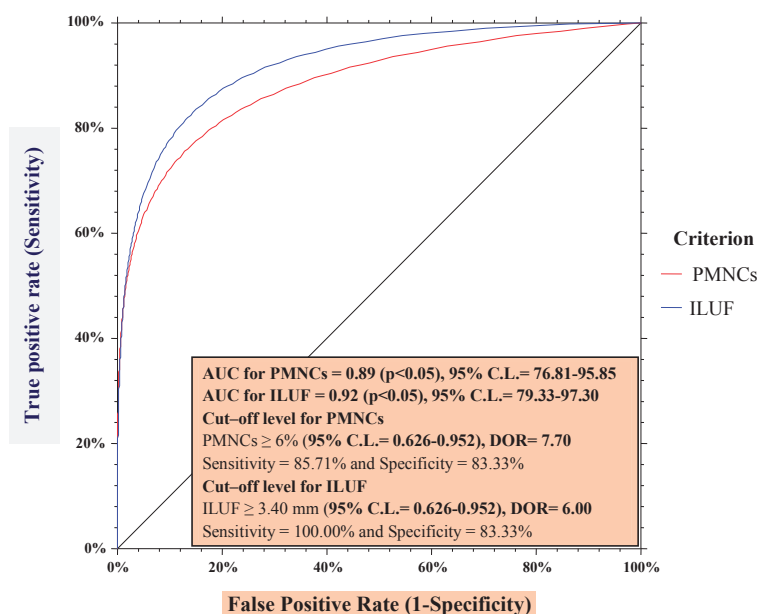Sensitivity = 100.00% and Specificity = 83.33%

Fig. 3. Receiver operator characteristics (ROC) curve for determining threshold level of PMNCs and ILUF in aiding the diagnosis of cytological endometritis in dairy cows at 8 weeks post-partum

On the basis of endometrial cytology, the incidence of subclinical endometritis in cows with a percentage of PMNCs above the threshold level was 48.89% whereas it was 55.56% in cows diagnosed with ILUF above the threshold level (Table 1 and 2). On combining semi- and non-invasive techniques, i.e. endometrial cytology and transrectal ultrasonography, the incidence was reported to be 40% (Table 2).

The number of days taken for completion of uterine involution (UINV) and the first post-partum ovulation (FPO) were significantly higher (P<0.01-0.05) in cows diagnosed with subclinical endometritis, based on both the diagnostic parameters, i.e. PMNCs percentage and ILUF, at 8 weeks postpartum. According to these findings, the number of cows ovulating within the first 4 weeks after parturition was numerically higher when both the diagnostic parameters were below the threshold level (Tables 1 and 2).

Table 1. Days taken to complete uterine involution and first post-partum ovulation in dairy cows (N=45) based on different PMNCs percentages (%) at 8 weeks post-partum (mean ± S.E.)

PMNCs percentagen (%)	Average PMNCs (%)	Uterine involution, d	First post-partum ovulation, d	Number (%) of cows ovulated within 4 weeks post-partum
<6% (n=23)	1.26 ± 0.39[b]	28.91 ± 1.10[b]	34.70 ± 3.10[y]	13 (56.52%)
≥6% (n=22)	8.32 ± 0.74[a]	34.41 ± 1.21[a]	45.82 ± 3.05[x]	4 (18.18%)

[a,b] Values with different superscripts within the same column are significantly different (P<0.01)
[x,y] Values with different superscripts within the same column are significantly different (P<0.05)

Table 2. Days taken to complete uterine involution and first post-partum ovulation in dairy cows (N=45) based on the ILUF level (mm) during trans-rectal ultrasonography at 8 weeks post-partum (Mean±S.E.)

ILUF level	Average PMNCs (%)	Uterine involution, d	First post-partum ovulation, d	Number (%) of cows ovulated within 4 weeks post-partum	Number (%) of cows having PMNCs ≥6%
<3.40 mm (n=20)	1.23 ± 0.29[b]	29.05 ± 1.27[y]	31.85 ± 3.23[b]	14 (70%)	4 (20%)
≥3.40 mm (n=25)	6.52 ± 0.46[a]	32.76 ± 1.12[x]	46.76 ± 2.61[a]	3 (12%)	18 (72%)

[a,b] Values with different superscripts within the same column are significantly different (P<0.01)
[x,y] Values with different superscripts within the same column are significantly different (P<0.05)

Linear regression and correlation analysis showed a significant correlation (r=0.28-0.42; P<0.01-0.05) between PMNCs percentage, UINV and FPO (Figure 4 and 5). Linear regression and correlation analysis also recorded a highly significant (r=0.54-0.88; P<0.01) correlation between both the diagnostic parameters, i.e. the PMNCs percentage increased along with the increase in ILUF (Figure 6).
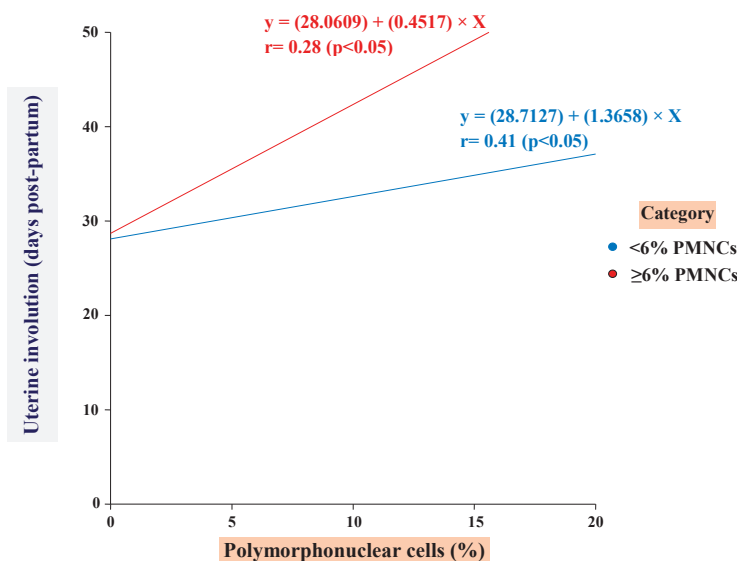

Fig. 4. Correlation between days taken for completion of uterine involution and PMNCs proportion (%) in dairy cows at 8 weeks post-partum
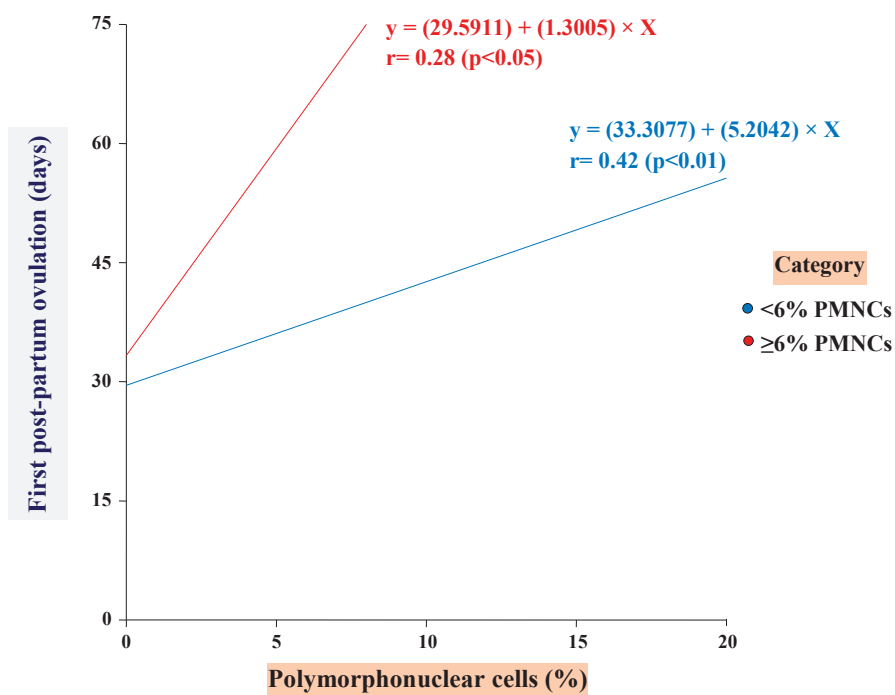
Fig. 5. Correlation between days taken for first post-partum ovulation and PMNCs proportion (%) in dairy cows at 8 weeks post-partum (based on ROC curve)
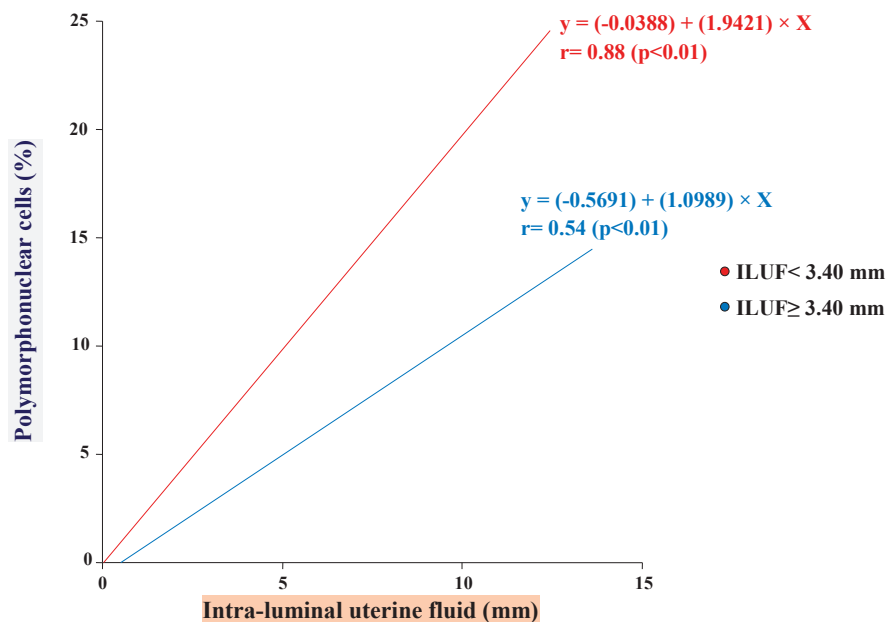

Fig. 6. Correlation between amount of intra-luminal uterine fluid (mm) and polymorphonuclear cells (%) in dairy cows at 8 weeks post-partum

Discussion

Cows with subclinical endometritis do not show any clinical signs for diagnostic purposes although it has continually affected the economics of the dairy industry in a substantial way for the last decade (WAGENER et al., 2017). For diagnosis of subclinical endometritis, the cytotape technique of endometrial cytology has been frequently used, as it offers more cellular detail and high specificity (PASCOTTINI et al., 2016). The cytotape-based incidence of subclinical endometritis has been reported to vary between 18.75-27.86%, which does not show any similarity with the findings of the present study (PASCOTTINI, 2016; RANA, 2019). However, the cut-off value for diagnosis of subclinical endometritis is still under discussion as it varies within a wide range due to different levels of immune response and the PMNCs population when uterine contamination occurred during the process of parturition (POTHMANN et al., 2019). On the basis of ROC curve analysis, PASCOTTINI (2016) and LEE et al. (2018) determined a PMNCs threshold range between 5-10% with a high detection rate of truely negative cows, i.e. 81.7-88.6% specificity, after 6 weeks of parturition, which is not akin to our findings.

Considering the importance of the postpartum period, an early diagnosis of subclinical endometritis, based on a non-invasive technique, is very helpful in determining the future fertility of dairy cows (TONI et al., 2015; VINITA et al., 2018). The B-mode of trans-rectal ultrasonography, a cow side test, detects the amount of fluid inside the uterine lumen (MEIRA et al., 2012), a thickened endometrial wall (PUROHIT et al., 2013), and cervical measurement ($\geq$5.0 cm; SALAH and YIMER, 2017) for ascertaining uterine inflammation, in the absence of any clinical signs of illness 4-5 weeks after parturition (SILPER et al., 2016). Similar to our findings, a level of $\geq$5.0 mm ILUF has been reported to be associated with the occurrence of subclinical endometritis at 6-7 weeks postpartum (DOUREY et al., 2011). At present, no threshold level of ILUF has been established for diagnosis of postpartum subclinical endometritis in dairy cows. An incidence (50%) similar to

the present study was reported for subclinical endometritis, but it was not validated for sensitivity and specificity (SHARMA and SINGH, 2019).

Various important events, such as uterine involution, endometrial regeneration and resumption of ovarian activity, occur during the post-partum period, and their occurrence is mainly affected by persistent uterine infections, i.e. clinical and subclinical endometitis (COTO and LUCY, 2018). The impact of subclinical endometritis on early postpartum reproductive parameters has been documented by various researchers, as it leads to a delay in the number of days to completion of UINV and FPO (DOUREY et al., 2011). Inhibition of estradiol production from aromatization of androgens often leads to alteration in the lifespan of the follicle at recruitment and selection, and consequently ovulation, which is mainly induced by persistent uterine infections (WILLIAMS et al., 2007). Any delay in early resumption of ovarian activity and an associated decrease in estradiol concentrations can disrupt the inherent defense mechanisms, as it decreases leukocyte migration to the uterus and hinders the rapid clearance of the inflammatory reaction (SUBANDRIO et al., 2000).

Many researchers have validated a correlation between the amount of intraluminal uterine fluid (ILUF) and the percentage of PMNCs diagnosed through trans-rectal ultrasonography and cytology, thus making ILUF a potential diagnostic marker for subclinical endometritis (DOUREY et al., 2011; MARINO et al., 2017). A uniform linear correlation (r=0.4-0.6) between intraluminal uterine fluid and the percentage of PMNCs was reported by SHARMA and SINGH (2019), which was slightly lower than in the present study.

In conclusion, the present study concluded the threshold levels of polymorphonuclear cells and intraluminal uterine fluid, thus aiding the diagnosis of subclinical endometritis after parturition in dairy cows. The combination of endometrial cytology and transrectal ultrasonography increased the diagnostic accuracy and helped in assessing the impact of subclinical endometritis on early post-partum reproductive parameters. In peroration, both the techniques can be highly useful in

acknowledging the threat of persistent uterine infections for the reproductive performance of cows reared in the ever-growing dairy industry.

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SAŽETAK

Cilj ovog rada bio je istražiti dijagnostiku supkliničkog endometritisa i njegov utjecaj na rane reproduktivne pokazatelje mliječnih krava (n = 45) u postpartalnom razdoblju. Količina polimorfonuklearnih stanica (PMNC u postotku) i intraluminalne maternične tekućine (ILUF u milimetrima) procijenjena je s pomoću test traka i transrektalne ultrasonografije kako bi se dijagnosticirao supklinički endometritis osam tjedana poslije porođaja. ROC krivulja upotrijebljena je kako bi se odredio dijagnostički prag PMNC-a i ILUF-a. Optimalna vrijednost praga za PMNC bila je ≥ 6 % (područje ispod krivulje (AUC) 0,89; osjetljivost 85,71 %; specifičnost 83,33 %; P < 0,05), a za ILUF ≥ 3,40 mm for (AUC – 0,92; osjetljivost 100 %; specifičnost 83,33 %; P < 0,05). Omjer dijagnostičkih izgleda (DOR) za razvoj supkliničkog endometritisa u krava koje su imale postotak PMNC-a i ILUF iznad praga bio je 7,70 za PMNC i 6,00 za ILUF. Analizirani su rani reproduktivni pokazatelji, kao što je broj dana potreban za završetak involucije maternice (UINV) i prva postpartalna ovulacija (FPO), te se pokazalo da su bili znakovito veći u krava sa supkliničkim endometritisom uzme li se u obzir postotak PMNC-a (P < 0,01 za UINV i P < 0,05 za FPO) i ILUF (P < 0,05 za UINV i P < 0,01 za FPO). Analiza linearne korelacije pokazala je znakovit odnos (P ≤ 0,01 – 0,05) između vrijednosti PMNC-a i ranih reproduktivnih pokazatelja. Slično, uočena je znakovita korelacija (P < 0,01) između ILUF-a i PMNC-a, bez obzira na dijagnostički prag. Endometralnom je citologijom i transrektalnom ultrasonografijom s dijagnostičkim pragom ≥ 6 % za PMNC i ≥ 3,40 mm za ILUF dijagnosticiran supklinički endometritis, zbog čega je odgođeno uspostavljanje uvjeta za ranu reprodukciju nakon porođaja.

Ključne riječi: dijagnostički prag; intraluminalna maternična tekućina; polimorfonuklerne stanice; reproduktivni pokazatelji; supklinički endometritis