Effect of elective cesarean section on the incidence of subclinical endometritis in Belgian Blue cows

Het effect van electieve keizersnede op de incidentie van subklinische endometritis bij Belgisch witblauwekoeien

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ABSTRACT

Over 95% of Belgian Blue (BB) cows deliver via elective cesarean section (CS). The aim of this study was to determine the effect of elective CS on the incidence of subclinical endometritis (SCE) using cytobrush (CB) and low-volume lavage (LVL) techniques and evaluate the sensitivity of both techniques in detecting SCE. Uteri of BB cows (n = 100) were collected from the slaughterhouse. Cytobrush samples followed by LVL cytology samples were collected from all uterine horns (n = 200). The samples collected by CB was rolled on a microscope slide while LVL samples were centrifuged, and the pelleted cells were then scattered over a microscope slide. In total, three hundred nucleated cells were identified and the proportion of polymorphonuclear (PMN%) to endometrial cells was assessed. The cut-off for SCE was set at ≥1% PMN. To compare the CS horn with its CS-free counterpart, linear (PMN%) and logistic regression (SCE positive versus SCE negative) modeling was performed. The PMN% in CB (0.34 ± 0.45%) samples was lower than in LVL (2.67 ± 0.45%) samples ($p < 0.0001$), suggesting LVL is a more sensitive technique than CB. The CS horn had no effect on the PMN% ($p = 0.99$ for CB and $p = 0.12$ for LVL), and hence on the incidence of SCE ($p = 0.18$ for CB and $p = 0.81$ for LVL).

SAMENVATTING

Meer dan 95% van de Belgisch witblauwe koeien kalft via een electieve keizersnede (CS). Het doel van het onderzoek was om de invloed van een electieve CS op de incidentie van subklinische endometritis (SCE) te bepalen met behulp van de cytobrush (CB)- en de laagvolumelavage (LVL)-technieken. Tevens werd de gevoeligheid van beide technieken voor het detecteren van SCE bepaald. De placenta's van Belgisch witblauwekoeien (n = 100) werden verzameld uit het slachthuis. Ctyobrush-stalen gevolgd door LVL-cytologiestalen werden genomen uit alle baarmoederhoornen (n = 200). De CB werd op een microscoopglaasje uitgerold. De LVL-stalen werden gecentrifugeerd, waarna de gepelleteerde cellen over een microscoopglaasje werden verspreid. Uiteindelijk werden driehonderd gekernde cellen geïndentificeerd, waarbij het aandeel polymorfonucleairen (PMN%) ten opzichte van de endometriumcellen werd bepaald. De grenswaarde voor SCE werd vastgesteld op ≥1% PMN. Om de CS-hoorn te vergelijken met zijn CS-vrije tegenhanger werden lineaire (PMN-percentage) en logistische regressiemodellen (SCE-positief versus SCE-negatief) opgesteld. Het PMN-percentage in de CB-stalen (0,34 ± 0,45%) was lager dan in de LVL-stalen (2,67 ± 0,45%) ($p < 0,0001$), wat suggereert dat de LVL-techniek gevoeliger is. Er kon geen verschil aangetoond worden tussen de beide uterushoornen wat het PMN-percentage betreft ($p = 0,99$ voor CB en $p = 0,12$ voor LVL). Zodoende kon er dus ook geen verschil in incidentie van SCE worden vastgesteld ($p = 0,18$ voor CB en $p = 0,81$ voor LVL).

INTRODUCTION

Cesarean section (CS) is an approach for managing challenging parturitions in cows, frequently used when dealing with fetal oversize or malposition, restricted uterine passage, and twinning (Newman and Anderson, 2005). Non-elective CS in the case of dystocia finds particular application within the context of dairy cows (Barkema et al., 1992a). In Belgium, a significant proportion of the cattle comprises double-muscled Belgian Blue (BB) cows. Given the distinctive physiological attributes of BB cows, characterized by a narrow pelvis in combination with oversized fetuses, the susceptibility to dystocia is heightened (Barkema et al., 1992b). Consequently, most BB calves are delivered via elective CS, which means that the CS method is selected as the first choice for delivery without any attempt for a parturition per vias naturales. This procedure has attained a high level of refinement in BB cows, marked by a rigorous protocol (Kolkman et al., 2007).

Subclinical endometritis (SCE) stands as a reproductive disorder in dairy cattle, characterized by inflammation of the endometrial lining devoid of overt clinical manifestations (Pascottini et al., 2023; Sadeghi et al., 2022; Sheldon et al., 2009). The employment of endometrial cytology, recognized for its simplicity and reliability, has been extensively leveraged for SCE diagnosis (Salah et al., 2017). This diagnostic approach hinges on the assessment of the polymorphonuclear cell proportions (PMN%) in endometrial cytology samples. Distinct cut-off values for PMN% are employed to diagnose SCE, contingent upon the postpartum day on which endometrial sampling is conducted (Wagener et al., 2017; Dubuc et al., 2010; Sheldon et al., 2006). Notably, two techniques, cytobrush (CB) and low-volume lavage (LVL), have been predominantly utilized to collect cytology samples (Salah et al., 2017; Pascottini et al., 2015; Kasimanickam et al., 2005). The CB technique entails the collection of samples from a confined uterine area, while the LVL technique harvests samples encompassing a larger surface of the endometrium (Van Schyndel et al., 2018; Pascottini et al., 2016). Previous studies have conducted comparative assessments of these two techniques, evaluating their effectiveness in endometrial cytology (Van Schyndel et al., 2018; Pascottini et al., 2015). Both approaches exhibit minimal invasiveness for the acquisition of adequate cellular material from the endometrial surface.

The genesis of SCE in dairy cows is often associated with risk factors like dystocia and retained placenta often coupled with metabolic disorders and immune dysfunction (Cheong et al., 2011; Salasel et al., 2010). In the case of BB cows, elective CS seems to emerge as a salient factor contributing to the potential onset of SCE (Barkema et al., 1992a), leading to lower pregnancy rates (20%-30% reduction). In the aftermath of elective CS, the intricate process of uterine involution, coupled with delayed wound remodeling, holds the potential to engender a state of persistent inflammation within the endometrial milieu (Sun et al., 2023) as depicted in Figure 1. Therefore, the authors hypothesize that delayed wound healing upon CS contributes to the development of SCE in BB cows. Thus, the objective of this study was to assess the effect of elective CS on the incidence of SCE in BB cows. To do so, a comparative approach was used between CB

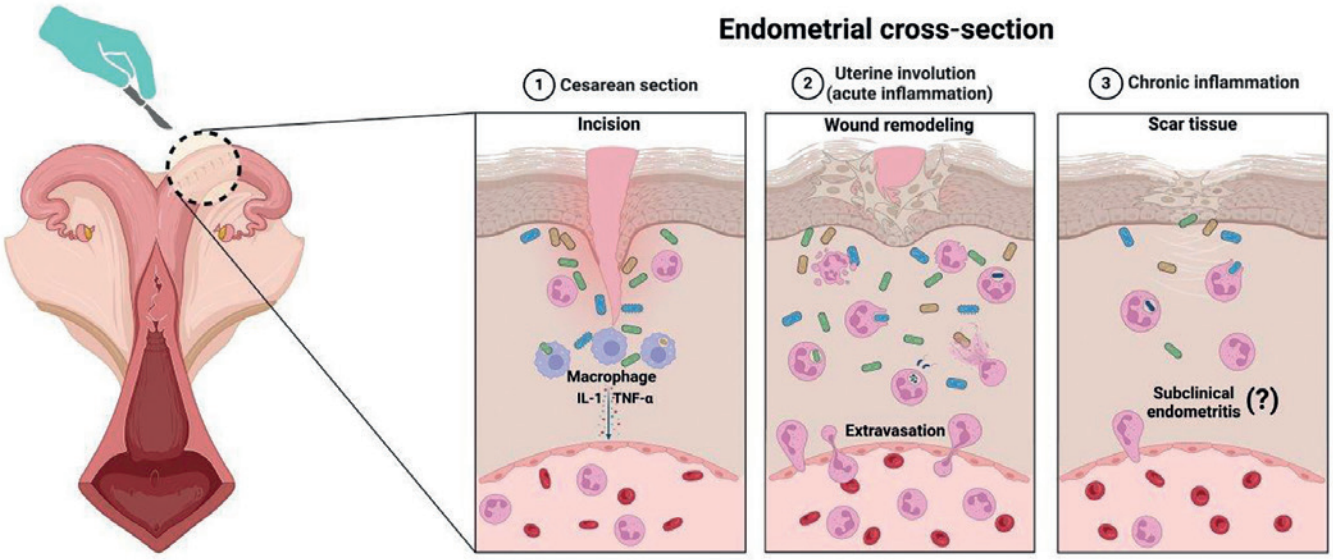

Figure 1. Schematic overview of the involution of the uterus after a cesarean section (CS) surgery. Following CS, the incision made in the uterine horn alongside placental detachment triggers inflammation and release of proinflammatory cytokines, notably interleukin-1 (IL-1) and tumor necrosis factor (TNF)-α (Sun et al., 2023). Subsequently, polymorphonuclear neutrophils respond to these molecular signals and migrate from the circulatory system to the uterine lining. This intricate process of uterine involution, coupled with delayed wound remodeling, can predispose for the development of uterine disease, including subclinical endometritis.

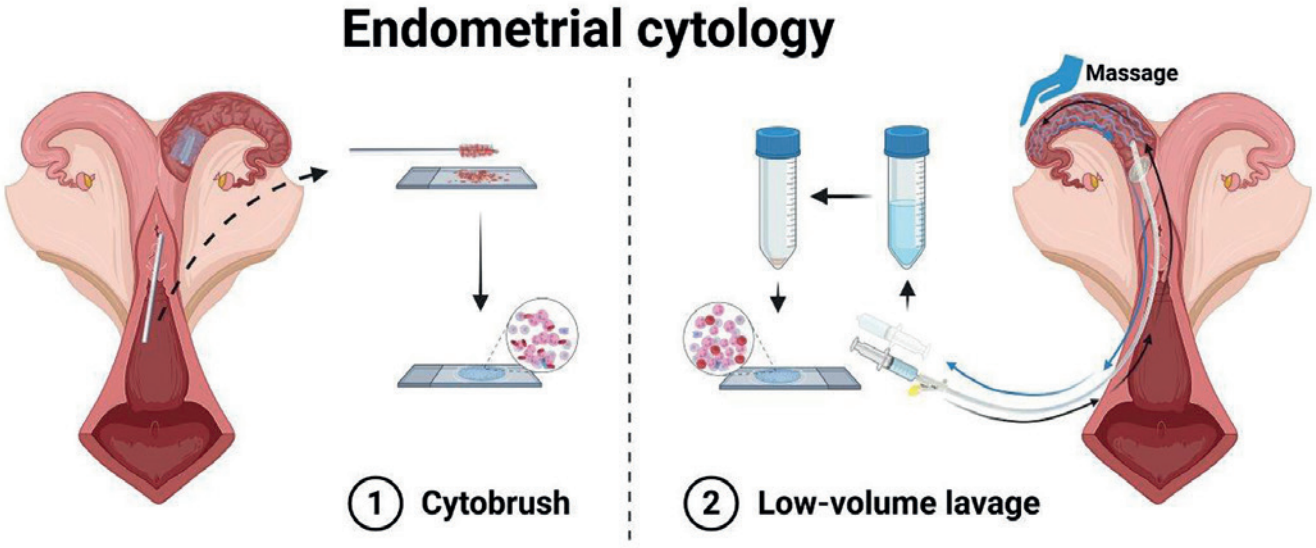

Figure 2. Schematic overview showing the techniques employed for collecting the endometrial cytology samples. The cytobrush was transcervically manipulated and once reaching the uterine horn, it was rotated twice, removed from the genital tract, and rolled on a microscope slide. Low-volume lavage followed cytobrush sampling. A Foley catheter was trancervically inserted into the uterine horn, 40 mL of sterile 0.9% saline solution was infused, followed by gentle massage for ten seconds. Then, the infused liquid within the uterine horn was collected and transferred into a 50 mL tube, which was centrifuged at 700 g for five minutes. The supernatant was discarded, and a drop of the pellet was placed on a microscope slide and gently spread over by another slide to distribute the cellular content of the drop.

and LVL techniques to independently collect ex vivo endometrial cytology samples from uterine horns of BB cows with versus without a CS scar.

MATERIAL AND METHODS

Animals and procedures

Genital tracts from one hundred BB cows were collected from a local slaughterhouse. Collected materials were transported at 4 °C within one hour after slaughtering, and sampling was performed upon arrival at the laboratory facilities. Uteri inclusion criteria were the absence of gross pathological signs of inflammation and the presence of an elective CS incision scar in either uterine horn. Before sampling, the uterine horn which had the latest elective CS scar incision was identified. Also, the presence of ovarian structures including a corpus luteum (CL) (≥ 1.5 cm) or a dominant follicle (FL) (≥ 8 mm) in either ovary was recorded.

Endometrial cytology collection by the cytobrush technique

The cytobrush technique was implemented to collect endometrial cytology samples (Figure 2), first from the left followed by the right uterine horn. Briefly, a Cytobrush Plus GT (Cooper Surgical, Berlin, Germany) was inserted into a sterile 22″ long equine infusion pipette (Agtech, Manhattan, KS, USA), which in its turn was encased within a 21″ long sanitary sheath (IMV, L'Aigle, France) to avoid contamination with cervical cells. The pipette was guided by a gloved hand and navigated through the cervix. Upon reaching the mid-part of the uterine horn, the sanitary sheath was punctured using the pipette tip to release the CB. The pipette was gently rotated twice, sampling in this way the dorsal part of the uterine horn in the area of the CS scar (if present). Finally, the CB was reintroduced into the pipette, and the device was carefully retracted back from the genital tract. Immediately, after the CB sampling, the brush's bristles were gently rolled on a microscope slide (Marienfeld, Lauda-Königshofen, Germany). This procedure was then repeated in the right uterine horn, in this way collecting a total of two hundred CB samples from one hundred uteri.

Endometrial cytology collection by the low-volume lavage technique

The low-volume lavage technique was performed after CB sampling (Figure 2). Samples were first collected from the left uterine horn followed by sampling of the right uterine horn. To perform LVL, a Foley catheter (Agtech Inc., Kansas, USA) directed by a metal guide was inserted into the reproductive tract via cervical manipulation. Upon reaching the mid-part of the uterine horn, the Foley's catheter balloon was inflated with 5 mL of normal saline to fixate it into the uterine horn lumen. Then, a 50-mL syringe (Terumo, Binan, Laguna, the Philippines) containing 40 mL of sterile 0.9% saline solution (Eurovet, Heusden-Zolder, Belgium) was connected to the Foley catheter. Following the infusion of the physiological solution, an additional 10 mL of air was introduced to effectively displace residual liquid through the pi-

pette. Next, a gentle massage of the uterine horn was conducted for ten seconds, facilitating uniform distribution of the infused liquid within the uterine horn. In the end, all the infused liquid within the uterine horn was recovered and transferred into a 50-mL falcon tube. This procedure was then repeated in the right uterine horn, in this way collecting a total of two hundred LVL samples from one hundred uteri. Following the LVL collection, the samples underwent centrifugation at 700 g for five minutes. The supernatant was discarded, and a drop of the pellet was placed on a microscope slide and gently spread over by another slide to distribute the cellular content of the drop.

Diff-Quik staining

Cytology slides (CB and LVL) were subjected to Diff-Quik staining (Diff-Quik, Fisher Diagnostics, Newark, DE, USA). Briefly, slides were immersed in Diff-Quik fixative for twenty seconds, after which excess fixative was removed. Subsequently, slides were subjected to Diff-Quik I for ten seconds, with the slides being gently moved up and down to facilitate even distribution of the stain. The slides were then placed in Diff-Quik II for an additional ten seconds, during which the slide was gently moved up and down. Subsequently, the slides were rinsed with water to eliminate excess stains and were then allowed to air-dry. Lastly, slides were mounted with Eukitt® Quick- hardening mounting medium (Sigma-Aldrich, Hoeilaart, Belgium).

Cytological evaluation and diagnosis of subclinical endometritis

All slides (n = 400) were examined using an optical light microscope (Kyowa Optical, Tokyo, Japan) under magnification of x40. Two experienced observers blind to each others' results analyzed the same slides to ensure reproducibility and minimize bias. The PMN-to-epithelial cell ratio was determined by assessing a total of three hundred nucleated cells per slide. Additionally, within ten high-power fields at a magnification of x40, slide quality (percentage of intact cells) and overall cellularity were assessed. Slide quality was appraised by calculating the percentage of intact cells, leading to the categorization of slides into two groups: good (> 50% intact cells) and bad quality (≤ 50% intact cells). For the assessment of total cellularity, cell numbers were estimated and categorized as low (≤ 100 cells per high power field), or high (>100 cells per high power field) (Rana et al., 2020; Pascottini et al., 2015) For the diagnosis of SCE, the cut-off was set at ≥1% PMN (Pascottini et al., 2017).

Statistical analyses

Manually collected data were exported to Microsoft Excel (Microsoft Corp., Redmond, WA), where data exploration and organization were done using the PivotTables function (Microsoft Excel). The statistical analyses were performed using R-core (version 4.0.4; R Core Team, Vienna, Austria). Linear and logistic regression models were built to identify factors associated with PMN% and the diagnosis of SCE at the individual horn level, respectively. The effect of the last CS scar (yes versus no), the sampling method (CB versus LVL), and their interaction were included as the fixed effects. Furthermore, the effect of the sampling method (CB versus LVL) was tested on the quality and cellularity of the cytology slides. Lastly, the presence of a FL or CL was also tested as a fixed effect to check their association with the incidence of SCE alongside CB and LVL sampling. For all the models, the uterine horn side (left or right) nested within the uterus ID was set as a random effect. Model residuals were assessed using a scatterplot of the studentized residuals for homoscedasticity, a linear predictor for linearity, and a Shapiro-Wilk test for normality. Differences between levels of explanatory variables were assessed with Tukey's post hoc test. The results are expressed as least squares mean and standard errors. Significance and tendency were declared at $P < 0.05$ and $0.05 < P < 0.1$, respectively.

RESULTS

Effect of the sampling technique on the proportion of polymorphonuclears and the incidence of subclinical endometritis

The results are shown in Figures 3A and 3B. Based on the collection of the four hundred cytology samples (n = 200 for CB and n = 200 for LVL), the PMN% in CB samples (0.34 ± 0.45%) was lower than in LVL samples (2.67 ± 0.45%; P < 0.0001) (Figure 3A). However, the incidence of SCE in CB samples (17 ± 3.2%) only tended to be lower than in LVL samples (23.6 ± 3.6%; P = 0.10) (Figure 3B).

Effect of elective cesarean section on the proportion of polymorphonuclears and the incidence of subclinical endometritis

The results are demonstrated in Figures 4A and 4B. For the CB samples, the comparison between the CS (0.48 ± 0.64%) versus the no-CS horns (0.22 ± 0.6%) revealed no differences in PMN% (P = 0.99). Similarly, between the CS (1.66 ± 0.64%) and no-CS horns (3.58 ± 0.61%), there was no difference in PMN% (P = 0.12) when sampling by LVL. Interestingly, when compared to CB, the PMN% retrieved via LVL was higher in the no-CS horns (P = 0.0004), while it was not different in the CS horns (P = 0.53) (Figure 4A).

For the CB samples, the incidence of SCE was 22.8 ± 4.9% in CS horns and 11.9 ± 3.3% in no-CS horns (P = 0.18). In the LVL samples, the incidence of SCE was 20.7 ± 4.7% in the CS horns while it was 26.2 ± 4.9% in the no-CS horns (P = 0.81). In the no-CS

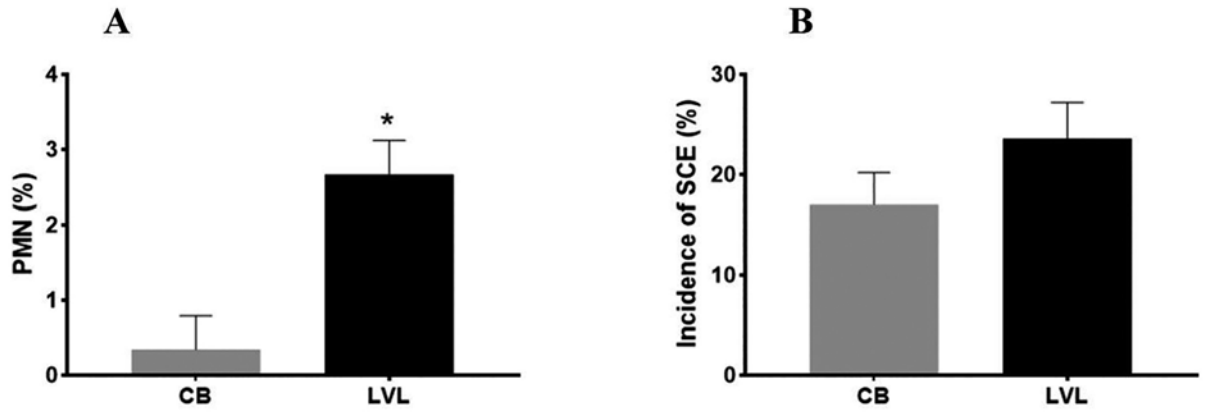

Figure 3. Effect of the sampling technique on the proportion of polymorphonuclears (PMN%) and the incidence of subclinical endometritis (SCE). Totally, four hundred cytology samples (n = 200 for cytobrush (CB) and n = 200 for low-volume lavage (LVL)) were collected. (A) The PMN% evaluation in CB and LVL techniques, (B) The incidence of SCE in CB and LVL techniques. The statistical significance is indicated as follows: * represents p < 0.05.

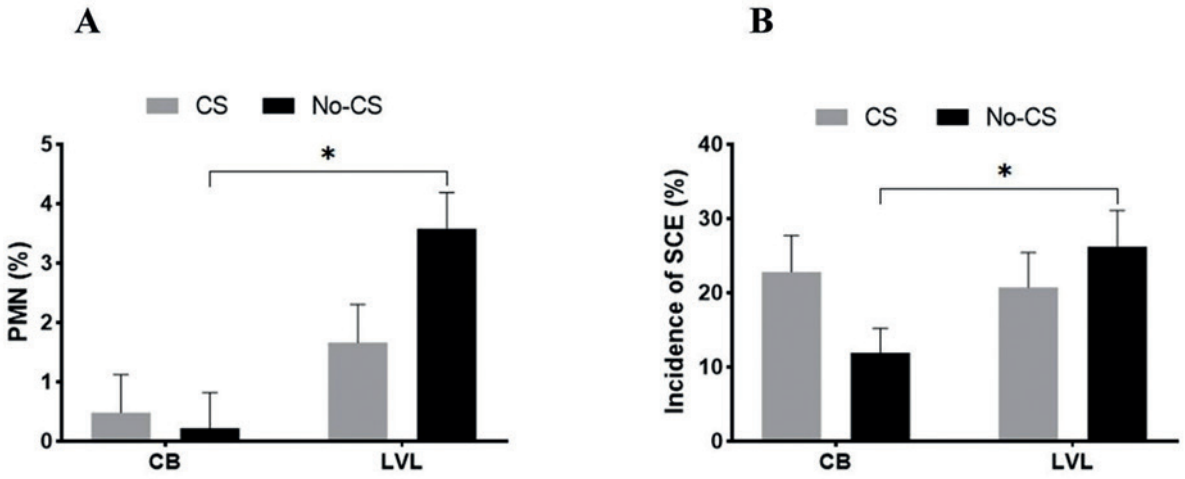

Figure 4. Effect of elective ceaserean section (CS) on the proportion of polymorphonuclears (PMN%) and the incidence of subclinical endometritis. (A) The interplay between elective CS and the diagnostic technique (cytobrush (CB) and low-volume lavage (LVL)) on PMN%, (B) The interplay between elective CS and the diagnostic technique (CB and LVL) on SCE%. The statistical significance is indicated as follows: * represents p < 0.05.

horns, the incidence of SCE was higher via LVL than via CB sampling (P = 0.04). However, in the CS horns, no differences in the incidence of SCE were found between the CB and LVL techniques (P = 0.98) (Figure 4B).

Effect of the sampling technique on quality and cellularity in the cytology slides

Representative images of endometrial cytology quality and cellularity, associated with the CB and LVL techniques are shown in Figure 5. Low-volume lavage sampling collected cells showed lesser quality (86.6 ± 3.8%) in comparison to CB (99 ± 0.69%; P < 0.0001). Both in CB as well as in LVL sampling, very few samples had low cellularity (0.37 ± 0.58 and 0.058 ± 0.11%, respectively), which did not allow for statistical inference.

Effect of ovarian structures on the incidence of subclinical endometritis

The results are depicted in Figures 6A and 6B. To study the effect of CL and FL on the incidence of SCE, comparisons were made at the individual horn level. For CB sampling, when a CL was present, the incidence of SCE was 18.9 ± 7.7%, while in the absence of a CL, it was 16.4 ± 3.4% (P = 0.98). Likewise, the incidence of SCE showed no difference when a FL was present (7.3 ± 7.3%) or not (17.8 ± 3.3%, P = 0.79) (Figure 6A). For LVL sampling, when a CL was present, the incidence of SCE was 33.8 ± 9.8%, while

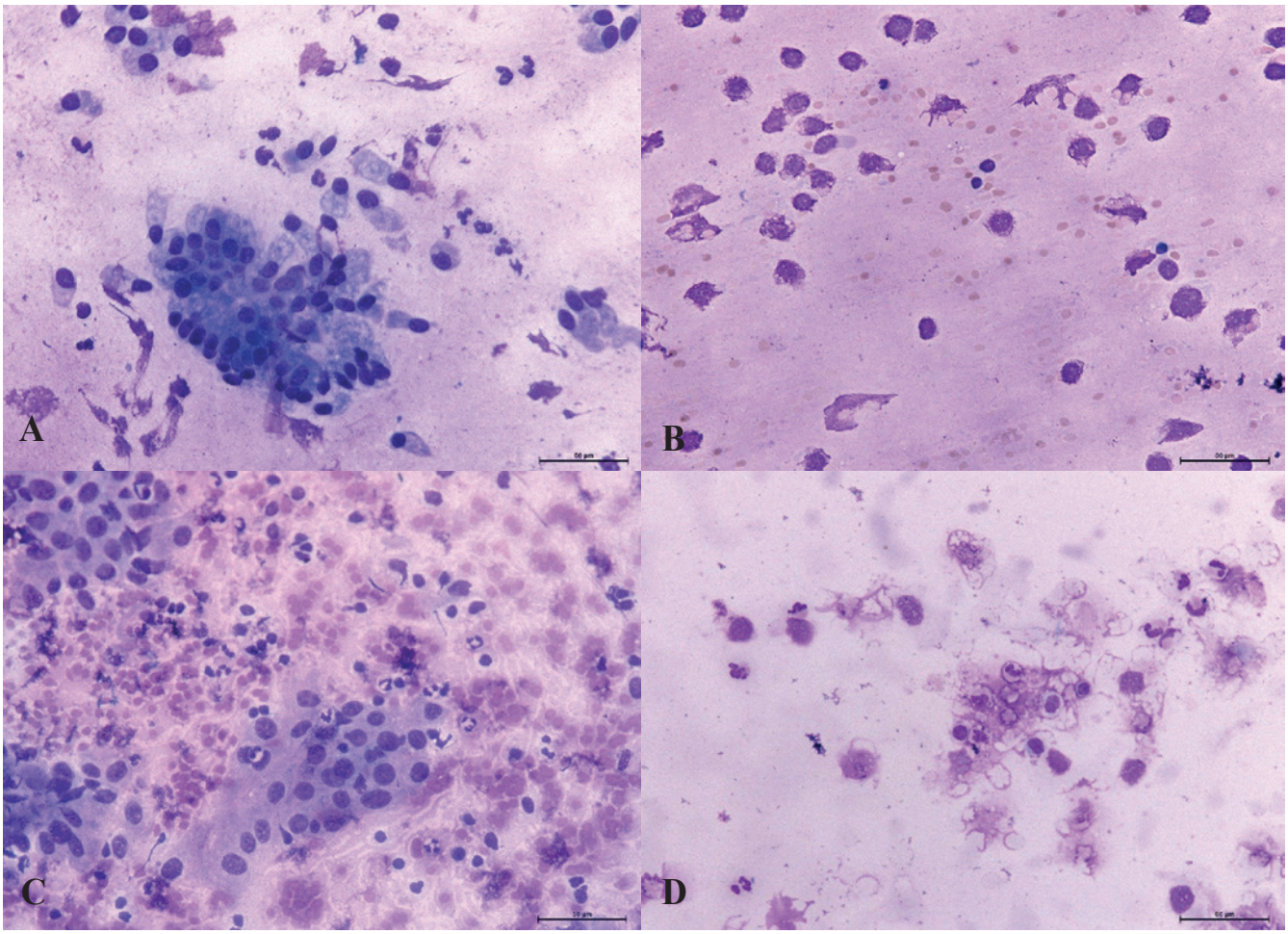

Figure 5. Endometrial cytology smears obtained by cytobrush and low-volume lavage techniques, stained with Diff-Quik and observed under the light microscope (x40). (A) Cytobrush sample scored with good quality (> 50% intact cells). (B) Low-volume lavage sample scored with bad quality (≤50% intact cells). (C) Cytobrush sample scored with high cellularity (>100 cells per high power field). (D) Low-volume lavage sample scored with low cellularity (≤100 cells per high power field).

it was 21.6 ± 3.9% in the absence of a CL (P = 0.58). Similarly, the incidence of SCE was not affected by the presence (23.4 ± 12.9%) or absence of a FL (23.7 ± 3.8, P = 0.99) (Figure 6B).

DISCUSSION

The findings in the present study elucidate that elective CS has no effect on PMN% nor on the incidence of SCE, irrespective of the cytology technique used for sample collection. However, it was observed that the choice of sampling method markedly affected the PMN%, with a higher PMN% and incidence of SCE obtained via LVL compared to CB sampling.

The PMN% obtained by LVL was notably higher than that obtained through the CB technique, which is consistent with prior research findings (Van Schyndel et al., 2018). This distinction in PMN% can be attributed to the inherent characteristics of each technique. With cytobrush sampling, cells are collected from an endometrial surface region that is no larger than 1 cm^2, while with LVL, a larger endometrial area is rinsed (Van Schyndel et al., 2018; Pascottini et al., 2016). Generally, when covering a larger area, there are more chances to capture a greater variety of cellular elements, including free-floating PMN, which may contribute to the higher PMN% when using the LVL technique. The LVL technique has the advantage of providing a more representative sample of the overall uterine environment. In contrast, the CB technique represents only a specific area of the endometrial lining, which some authors considered as representative of the whole endometrial surface (Janowski et al., 2013; Kasimanickam et al., 2004). The above-mentioned statements are likely to explain variations in PMN% between the CB and LVL techniques. Furthermore, SCE is diagnosed based on PMN% cut-off points. Most SCE cut-off points, however, were created based on CB sampling (Wagener et al., 2017). Only in a few studies, a large number of samples were collected using the LVL technique to create a LVL-specific cut-off point for SCE (5% PMN at Week 6 (Gilbert et al., 2005); ≥ 10% PMN at Week 5, or ≥ 8%

PMN at Week 7 after calving (Galvão et al., 2011). Thus, SCE cut-off values created for different sampling techniques may not be interchangeably applied to each other. Collectively, the results of the present study underscore the importance of the chosen technique for endometrial cytology, which can significantly impact the research outcome.

Elective CS had no substantial impact on PMN% nor on the incidence of SCE in BB cows. However, this observation can be attributed to the fact that uteri were collected after the completion of uterine involution, signifying full recovery from the surgical incision. Furthermore, it is essential to consider that a meticulous and stringent CS protocol is implemented in BB cows, establishing it as the preferred method for delivery (Kolkman et al., 2007), which effectively minimizes the risk of postpartum uterine diseases. Additionally, when compared to dairy cows, in which SCE affects around 20 to 30% of animals (Salasel et al., 2010; LeBlanc, 2008; Gilbert et al., 2005), factors like high milk production, metabolic disturbances, and concomitant immune dysfunction, all significantly contribute to the genesis of SCE (Pascottini et al., 2023; Berry et al., 2014). Belgian Blue cows experience these issues to a much lesser extent (if at all), resulting in a lower incidence of SCE as observed in the present study in comparison to studies performed in dairy cows. The incidence of SCE can also be associated with the days postpartum when samples are collected alongside the PMN% cut-off point employed (Barański et al., 2012). Generally, a PMN threshold of 10 to 18% is utilized when cytology samples are obtained between 21 and 33 days postpartum, while 5 to 10% PMN is employed for samples collected within days 34 to 47 after calving (Wagener et al., 2017; Sheldon et al., 2006). These variations often result in inconsistencies in SCE definition and thus in prevalences among studies (Pascottini et al., 2015; Barlund et al., 2008). For the present study, genital tracts were procured from the slaughterhouse and only uteri with visibly complete involution were selected. In light of this situation, a cut-off PMN value of ≥1% was established as the threshold for defining SCE. This threshold aligns with the one used by Pascottini et al. (2017), where the samples were collected after the end of the voluntary waiting period and the applied threshold was based on a receiver operator characteristic curve analysis. In the present study, the precise calving dates of the collected post-mortem uteri were not available, which the authors recognize as a limitation. Thus, it is possible that results obtained at different time points, such as earlier post-delivery sampling, may differ from those obtained at the time of complete uterine involution, which is due to the fact that uterine and immune processes undergo changes over time. On the other hand, it was aimed to assess whether SCE is more prevalent in fully involuted CS-horns than in their CS-free counterparts, which could contribute to the well-known lower pregnancy rates in cows that have delivered via CS. Therefore, it was not necessary to know the exact calving dates of the cows included in the present study.

There were discernible variations in the slide quality between CB and LVL techniques. Interestingly, the CB sampling exhibited a higher percentage of intact cells, whereas smears obtained through the LVL technique were characterized by more distorted cells. Comparable findings have been reported by other researchers, underscoring the higher incidence of distortion in cells obtained via the LVL technique, likely attributed to prolonged liquid recovery collection intervals and the centrifugation process (Rana et al., 2020; Barlund et al., 2008; Kasimanickam et al., 2005). Samples collected by both techniques were characterized by adequate cellularity for proper cytologic evaluation. No significant associations were

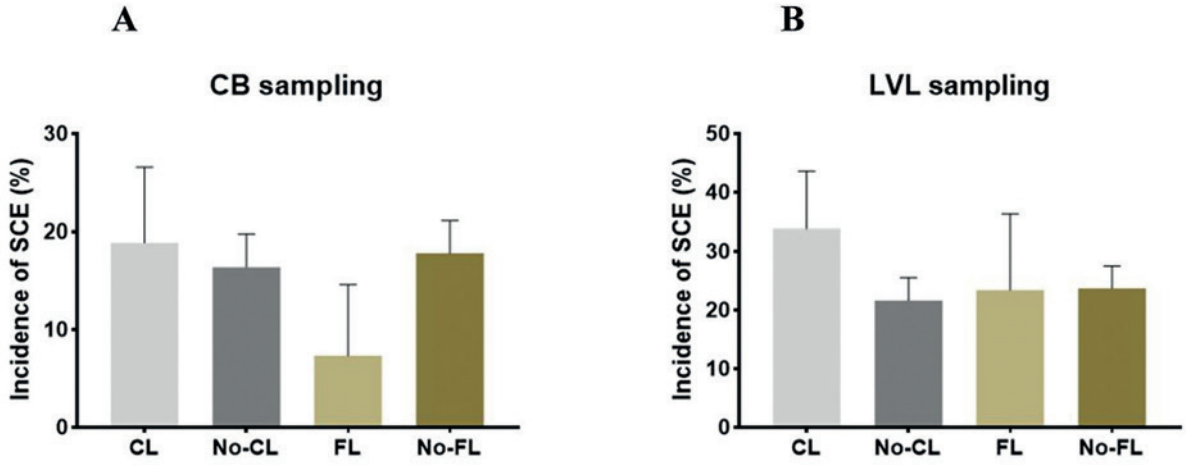

Figure 6. Effect of ovarian structures including a corpus luteum (CL) (≥ 1.5 cm) or a dominant follicle (FL) (≥ 8 mm) on the incidence of subclinical endometritis (SCE%). (A) The effect of CL or FL on SCE% based on cytobrush (CB) samples. (B) The effect of CL or FL on SCE% based on low-volume lavage (LVL) samples.

found between the presence of a CL or dominant FL and the occurrence of SCE in the present study, which aligns with previous investigations performed in dairy cows (Carneiro et al., 2014).

The present study demonstrates that elective CS does not have an impact on the incidence of SCE in BB cows at the timepoint of complete uterine involution, irrespective of the sampling technique used to collect the endometrial specimens. However, further research is needed to determine whether this holds true at earlier timepoints post delivery. The higher PMN% observed in LVL compared to CB samples may be ascribed to the broader uterine horn surface area covered during the flushing procedure. In this study, comprehensive insights are provided into the intricate interplay between elective CS, the incidence of SCE, and the diagnostic efficacy of CB and LVL within the realm of endometrial cytology in cows.

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