DIAGNOSIS OF SUBCLINICAL ENDOMETRITIS AND SUBSEQUENT REPRODUCTIVE STATUS IN POSTPARTUM CATTLE

By

SALAH NOORI MOHAMMED

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

May 2018

DEDICATION

THIS THESIS IS DEDICATED TO MY FATHER AND IN MEMORY OF MY LATE MOTHER AND TO MY FAMILY WITH LOVE AND RESPECT

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Doctor of Philosophy

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Chairman : Nurhusien Yimer Degu, PhD
Faculty : Veterinary Medicine

Endometritis is one of the most common diseases that affect reproductive performance in dairy and beef cows (Sheldon *et al*., 2006). There is lack of studies about occurrence of subclinical endometritis and its diagnosis using cytological methods in Malaysia. Moreover, failure of response to antibiotics by pathogenic bacteria in uterine infections possibly due to drug resistance warranted the need to conduct this study.

The aims of this study were: 1) to evaluate three different methods of collection of endometrial cytological samples, 2) to determine the agreement among bacteriological findings, vaginal discharges, and endometrial cytology for endometritis detection in postpartum beef cows, 3) to determine the occurrence of subclinical endometritis (SCE) in postpartum beef cows, to compare the ovarian activity between SCE and healthy cows , 4) to assess the interaction between SCE cows and selected cytokines, acute phase proteins (APPs) and 5) to evaluate the agreement between endometrial cytology and ultrasound examination for diagnosis of SCE in postpartum beef cows. All parametric data after testing by the Shapiro–Wilk test were analyzed using t-test, one-way ANOVA as well as Tukey and Duncan post hoc tests at a probability threshold, $P < 0.05$. For non-parametric data, Kruskal-Wallis and Mann-Whitney tests were performed; all by using SPSS software.

Three different methods to collect cytological endometrial samples cotton swab (CS), cytobrush (CB) and low volume flush (LVF) were used to determine the mean of polymorphonuclear leukocytes (PMN) under high power field (HPF) microscopy (400x). Forty beef cows aged 3 to 7 years were subjected for cytological sampling at week three and four postpartum using the above methods. The mean number of

PMN from CB alone was significantly higher (11.3 ± 0.53 cells HPF^{-1}) than CS (7 ± 0.37 cells HPF^{-1}) and LVF (6 ± 0.35 cells HPF^{-1}) methods. Smears from CB had more endometrial cells and PMN (58.55 ± 1.41 cells HPF^{-1}), which were significantly higher ($P < 0.05$) than CS and LVF methods. Both CB and CS methods yielded significantly more intact PMN and endometrial cells (62.4 % and 61.9 %) than LVF (52.4 %). In conclusion, CB was found to be better and effective technique compared with the other cytological methods.

For objective 2, a total of 82 postpartum beef cows at 20–30 days post-calving, were used in this study. All the cows were examined by transrectal palpation and vaginal secretions collection. Endometrial swab samples for cytology and bacteriology were collected using CB. A four-grade system (0 = clear mucus, 1 = mucus containing flecks of pus, 2 = discharge including < 50% pus, and 3 = involving > 50% pus) was used to categorize vaginal secretions of these cows. Of the total 82 cows studied, 11% (9/82) had grade 1-3 vaginal secretions and indicated to have clinical endometritis (CE), whereas nine of the 73 clinically healthy cows (12.32%) were diagnosed with subclinical endometritis (SCE ≥ 8 % PMN). *Escherichia coli* was the most common bacteria isolated from SCE (42%), and CE cows (38%), which were significantly higher ($P<0.05$) than healthy cows (14.6%). The antimicrobial sensitivity test assessed based on the inhibition effects on in vitro bacterial growth showed that most of the isolated bacteria were sensitive to enrofloxacin and tetracycline.

To compare ovarian activity between cows with SCE and healthy cows, and also to determine the interaction between SCE and selected proinflammatory cytokines (IL-6 and IL-8), and acute phase proteins (APPs) like haptoglobin (Hp). A total of 96 postpartum beef and 52 Friesian Sahiwal dairy cows were used. All postpartum cows were checked by transrectal palpation weekly beginning from week 3 until week 16 to evaluate uterine involution, and resumption of ovarian activity by detecting growing follicles on both ovaries. Endometrial samples were collected using CB technique between day 22 and day 28 after calving to identify cows with SCE. The occurrence of SCE was higher 15.3% (8/52) in a dairy group than the beef group 12.5% (12/96) at week 4 postpartum. Twelve beef and 8 dairy healthy cows were randomly selected as control to compare with cows diagnosed with SCE. Blood samples were collected from SCE and healthy cows from week 3 until week 7 to check the level of serum progesterone, IL-6, IL-8 and Hp. Results showed prolonged postpartum anestrus in postpartum beef cows mainly associated with cessation of ovarian activity, leading to increased days open. Progesterone concentration was less than 1 ng/mL in both SCE and healthy beef cows. In dairy cows, the resumption of ovarian activity was faster in healthy cows (20.5 ± 0.9 days) than SCE (37.1 ± 0.7 days) postpartum and the interval from calving to first ovulation was significantly shorter in healthy cows (29.4 ± 0.7 days) than cows with SCE (47.5 ± 0.9 days). Results revealed elevated levels of proinflammatory cytokines (IL-6 and IL-8) in cows with SCE ($P < 0.05$) compared with healthy cows during week 4 -7 postpartum in both beef and dairy groups. The level of Hp in beef and dairy group was higher ($P < 0.05$) in cows with SCE than healthy cows during most of the weeks of 4 -7 postpartum periods.

Lastly, using ultrasonography as a diagnostic tool to diagnosis endometritis compared with endometrial cytology method was evaluated in 53 postpartum beef cows. The study was conducted between day 20 and day 35 postpartum (at week 4 and 5) postpartum using ultrasound and CB endometrial examination methods to diagnose endometritis. Results showed that the ultrasound method is a useful and practical tool to diagnose endometritis, especially when it is combined with evaluation of intrauterine fluid accumulation and the cervical diameter ($\geq$ 5 cm).

Overall, the study revealed that prevalence of SCE in cows was low and cytobrush method was found to be superior and effective technique to obtain endometrial cytological samples. *E. coli was* the major risk factor found associated with SCE in beef cows. The antimicrobial sensitivity test showed that most of the bacteria isolated were sensitive to enrofloxacin and tetracycline. The levels of IL-6, IL-8, Hp can use as diagnostic markers for SCE as long as these cows are without clinical diseases and not exposed to stress factors. The ovarian activity was faster significantly in healthy dairy cows than endometritis cows. Prolonged postpartum anestrus was the common cause to increased calving-to-conception interval and impaired beef reproductive performance.

Abstrak tesis yang disampaikan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

DIAGNOSIS SUBKLINIKAL ENDOMETRITIS DAN STATUS REPRODUKTIF SELANJUTNYA DALAM LEMBU PASCA BERSALIN

Oleh

SALAH NOORI MOHAMMED

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Endometritis adalah salah satu penyakit paling biasa yang mempengaruhi prestasi pembiakan dalam lembu tenusu dan lembu pedaging (Sheldon et al., 2006). Terdapat kekurangan kajian tentang kejadian endometritis subklinikal dan penggunaan kaedah sitologi untuk mendiagnosisnya di Malaysia. Selain itu, kegagalan tindak balas terhadap antibiotik oleh bakteria patogen dalam jangkitan rahim mungkin disebabkan oleh rintangan dadah yang menjamin keperluan untuk menjalankan kajian ini.

Tujuan kajian ini adalah: 1) untuk menilai tiga kaedah pengumpulan sampel sitologi endometrium, 2) untuk menentukan kesepakatan antara penemuan bakteriologi, lelehan vagina, dan sitologi endometrium untuk pengesanan endometritis dalam lembu pedaging postpartum, 3) untuk menentukan kejadian endometritis subklinikal dalam lembu pedaging postpartum, membanding aktiviti ovari dan keseimbangan tenaga antara endometritis subklinikal (SCE) dan lembu yang sihat semasa tempoh selepas bersalin, 4) untuk menilai interaksi antara lembu SCE dan sitokin terpilih, protein fasa akut (APP) dan 5) untuk menilai persetujuan antara sitologi endometrium dan pemeriksaan ultrasound untuk diagnosis SCE dalam lembu pedaging postpartum.

Tiga kaedah yang berbeza untuk mengumpul sampel endometrium sitologi (swab kapas, CS, cytobrush, CB dan flush volume rendah, LVF) digunakan untuk menentukan purata polimorfonuklear leukosit (PMN) di bawah mikroskop medan kuasa tinggi (HPF) (400x). Empat puluh ekor lembu pedaging berumur 3 hingga 7 tahun tertakluk kepada sampel sitologikal pada minggu ke tiga dan empat selepas bersalin menggunakan kaedah di atas. Jumlah purata PMN dari CB sahaja jauh lebih

tinggi (11.3 ± 0.53 sel HPF^{-1}) daripada kaedah CS (7 ± 0.37 sel HPF^{-1}) dan LVF (6 ± 0.35 sel HPF^{-1}). Smear dari CB mempunyai lebih banyak sel endometrium dan PMN (58.55 ± 1.41 sel HPF^{-1}), yang jauh lebih tinggi ($P < 0.05$) daripada kaedah CS dan LVF. Kedua-dua kaedah CB dan CS menghasilkan PMN dan sel endometrium yang lebih utuh (62.4% dan 61.9%) daripada LVF (52.4%). Sebagai kesimpulan, CB didapati lebih baik dan berkesan berbanding dengan kaedah sitologi yang lain.

Untuk objektif 2, sebanyak 82 ekor lembu pedaging postpartum pada 20-30 hari pascaberanak, telah digunakan dalam kajian ini. Semua lembu diperiksa oleh palpasi transrectal, dan koleksi rembesan faraj. Sampel swab endometrial untuk sitologi dan bakteriologi telah dikumpul menggunakan CB. Sistem empat gred (0 = lendir jelas, 1 = lendir yang mengandungi tompok-tompok nanah, 2 = lelehan termasuk <50% nanah, dan 3 = melibatkan > 50% nanah) digunakan untuk mengkategorikan rembesan faraj lembu-lembu ini. Daripada jumlah 82 ekor lembu yang dikaji, 11% (9/82) mempunyai rembesen faraj gred 1-3 dan menunjukkan mempunyai endometritis klinikal (CE), sedangkan sembilan daripada 73 lembu yang sihat secara klinikal (12.32%) didiagnosis dengan endometritis subklinikal (SCE , ≥ 8% PMN). *Escherichia coli* adalah bakteria yang paling biasa diasingkan dari SCE (42%) dan lembu CE (38%), yang jauh lebih tinggi ($P < 0.05$) daripada lembu yang sihat (14.6%). Ujian kesensitifan antimikrobial yang dinilai berdasarkan kesan perencatan pertumbuhan bakteria in vitro menunjukkan kebanyakan bakteria yang diasingkan sensitif terhadap enrofloxacin dan tetracycline.

Untuk membandingkan aktiviti ovari antara lembu yang menghidapi SCE dan lembu yang sihat, dan juga untuk menentukan interaksi antara SCE dan sitokin terpilih, protein fasa akut (APP). A sejumlah 96 daging lembu pasca lahir dan 52 lembu tenusu Friesian Sahiwal telah digunakan. Semua lembu postpartum diperiksa mingguan secara palpasi transrektum bermula dari minggu 3 hingga minggu ke-16 untuk menilai involusi rahim, simetri tanduk rahim dan penyambungan semula aktiviti ovari dengan mengesan folikel-folikel yang membesar pada kedua-dua ovari. Sampel endometrial dikumpulkan menggunakan teknik CB antara hari ke-22 dan hari ke-28 selepas anak lembu dilahirkan untuk mengenal pasti lembu dengan SCE. Kejadian SCE adalah lebih tinggi 15.3% (8/52) dalam kumpulan lembu tenusu daripada kumpulan lembu pedaging 12.5% (12/96) pada minggu 4 postpartum. Dua belas lembu pedaging dan 8 lembu tenusu yang sihat dipilih secara rawak sebagai kawalan untuk membandingkan dengan lembu yang didiagnosis dengan SCE. Sampel darah dikumpulkan dari SCE dan lembu yang sihat dari minggu 3 hingga minggu 7 untuk memeriksa tahap serum progesteron, IL-6, IL-8 dan haptoglobin (Hp). Hasil menunjukkan anestrus postpartum yang berpanjangan dalam lembu pedaging postpartum berkaitan rapat dengan pemberhentian aktiviti ovari, yang menyebabkan peningkatan hari terbuka. Kepekatan progesteron adalah kurang daripada 1 ng/ml dalam kedua-dua endometritis dan lembu sapi yang sihat. Walau bagaimanapun, kepekatan IGF-1 serum lebih tinggi dalam lembu yang sihat berbanding dengan lembu pedaging SCE walaupun tidak penting. Dalam lembu tenusu, pemulihan aktiviti ovari adalah lebih cepat pada lembu yang sihat (20.5 ± 0.9 hari) daripada SCE (37.1 ± 0.7 hari) postpartum dan tempoh diantara masa beranak

dan ovulasi pertama jauh lebih pendek pada lembu yang sihat (29.4 ± 0.7 hari) daripada lembu dengan SCE (47.5 ± 0.9 hari). Keputusan menunjukkan tahap sitokin proinflamasi yang meningkat (IL-6 dan IL-8) dalam lembu dengan SCE ($P < 0.05$) berbanding dengan lembu yang sihat pada minggu ke-4 hingga ke-7 postpartum dalam kedua-dua kumpulan lembu pedaging dan tenusu. Tahap Hp dalam kumpulan lembu pedaging dan tenusu adalah lebih tinggi ($P < 0.05$) dalam lembu dengan SCE daripada lembu yang sihat pada kebanyakan minggu 4 -7 tempoh postpartum.

Akhir sekali, menggunakan ultrasonografi sebagai alat diagnostik untuk mendiagnosis SCE berbanding kaedah sitologi endometrial telah dinilai pada 53 ekor lembu postpartum. Kajian ini dijalankan antara hari postpartum ke-20 dan ke-35 (pada minggu ke-4 dan 5) postpartum menggunakan kaedah pemeriksaan ultrabunyi dan pemeriksaan endometrium CB untuk mendiagnosis SCE. Keputusan menunjukkan bahawa kaedah ultrabunyi adalah kaedah yang berguna dan praktikal untuk mendiagnosis endometritis pada minggu ke-4 dan 5 postpartum, terutama sekali apabila digunakan bersama pernilaian pengumpulan cairan intrauterin dan diameter serviks (≥5 cm).

Secara keseluruhan, kajian menunjukkan bahawa kelaziman SCE dalam lembu pedaging adalah rendah dan kaedah cytobrush didapati merupakan teknik yang unggul dan berkesan untuk mendapatkan sampel sitologi endometrium. *E. coli* merupakan faktor risiko utama yang dikaitkan dengan SCE dalam lembu pedaging. Ujian kepekaan antimikrobial menunjukkan bahawa kebanyakan bakteria yang diasingkan sensitif terhadap enrofloxacin dan tetracycline. Tahap IL-6, IL-8, dan Hp adalah lebih tinggi dalam lembu dengan SCE berbanding dengan lembu yang sihat, menunjukkan potensi mereka sebagai penanda diagnostik untuk SCE. Aktiviti ovari adalah lebih cepat dalam lembu tenusu yang sihat daripada lembu pedaging yang sihat dan lembu endometritis. Anestrus postpartum yang berpanjangan adalah punca paling biasa meningkatkan kadar beranak kepada tempoh penghamilan dan prestasi pembiakan lembu pedaging.

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I certify that a Thesis Examination Committee has met on 16 May 2018 to conduct the final examination of Salah Noori Mohammed on his thesis entitled "Diagnosis of Subclinical Endometritis and Subsequent Reproductive Status in Postpartum Cattle" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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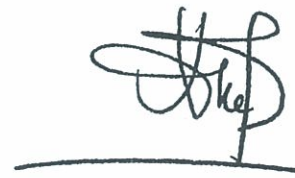

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LIST OF ABBREVIATIONS

AI	Artificial insemination
BA	Blood agar
BCS	Body condition score
BHBA	β-Hydroxybutyric acid
CB	Cytobrush
CE	Clinical endometritis
CI	Calving interval
CL	Corpus luteum
CS	Cotton swab
CTE	Cytological endometritis
DF	Dominant follicle
FSH	Follicle stimulate hormone
GnRH	Gonadotrophin releasing hormone
E coli	*Escherichia coli*
ELISA	Enzyme-Linked Immunosorbent Assay
Hp	Haptoglobin
HPF	High-power field
KK	Kedah-Kelantan
IL-6	Interleukin-6
IL-8	Interleukin-8
LH	Luteinizing hormone
LP	Luteal phase
LPS	Lipopolysaccharide
LVF	Low volume fluid
MCA	MacConkey Agar
NEB	Negative energy balance
NaCl	sodium chloride
NEFA	Nonesterified fatty acid
P4	Progesterone
PMN	Polymorphynuclus
PGF2α	Prostaglandin F2 alaph

RIA	Radioimmunoassay
rpm	Revolution per minute
SAA	Serum amyloid A
SCE	Subclinical endometritis
SPSS	Statistical package for social science
UPM	Universiti Putra Malaysia
US	Ultrasonography

CHAPTER 1

INTRODUCTION

Reproduction is considered as one of the most important features of cattle production. Studies have shown that many cows do not achieve peak reproductive performance and thus, result in a significant economic loss (Thatcher *et al*., 1996; LeBlanc *et al*., 2002; Galvao *et al*., 2010a). Approximately, 75% of postpartum disorders in cattle happen during the first 30 days after parturition (LeBlanc *et al*., 2006). There are many disorders and diseases like dystocia and uterine infection related to the postpartum period (McDougall, 2001; Sheldon *et al*., 2009), impairment of postpartum immune activity (Hammon *et al*., 2006), hormonal and metabolic alterations (Bauman and Currie, 1980; Yimer *et al*., 2010), energy balance (Drackley, 1999).

The uterus after calving is exposed to several types of microbial contamination and this causes many postpartum uterine diseases (Sheldon *et al*., 2006; Evans and Walsh, 2011). Metritis is an acute inflammatory reaction mostly due to bacterial infection of the uterus during the first few weeks after parturition. On the other hand, endometritis is a chronic condition of the cows due to incomplete clearance of the bacterial contaminants or due to persistent bacterial pathogens in the uterus (Sheldon *et al*., 2004). Clinically, it is manifested by a substantial increase in the number of polymorphonuclear leukocytes (PMN) in the uterus (Barlund *et al*, 2008) and has been classified into clinical and subclinical types (Sheldon *et al*., 2006). Clinical endometritis (CE) is associated with purulent or mucopurulent uterine discharge, 21 to 26 days postpartum. While the presence of more than 18% of polymorphonuclear (PMN) cells in uterine cytology samples collected 21–33 days postpartum or more than 10% PMNs in samples taken at days 34–47 is seen in cases of subclinical endometritis (SCE) (Kasimanickam *et al*., 2004). The incidence of CE and SCE is high and has been reported by many authors (Gilbert *et al*., 2005; Dubuc *et al*., 2010; Plöntzke *et al*., 2010; McDougall *et al*., 2011). For instance, more than 40% of cows were reported to have various degrees of uterine disease one-week post-calving. The incidence of reported cases, however, varied between 36-50% in cow farms surveys (Zwald *et al*., 2004). Additionally, about 20% of cows were reported to have suffered from systemic signs of metritis (Benzaquen *et al*., 2007) and more than 15% of cows that suffered from metritis developed clinical endometritis while about 30% converted to subclinical endometritis after three weeks post calving (Gilbert *et al*., 2005; Sheldon *et al*., 2006). In more than 90% of cows, several species of microorganisms can be isolated few weeks after parturition (Földi *et al*., 2006). However, the majority of the cows can clear these bacteria spontaneously (LeBlanc *et al*., 2002). Initial contamination of the uterus in the beginning of the postpartum period with *Escherichia coli* paves the way for subsequent infection with other bacteria or viruses (Donofrio *et al*., 2008). However, cases of acute endometrial lesions are basically caused by *Arcanobacterium pyogenes* (Sheldon *et al*., 2009) which is the most common prevalent microbial in the late postpartum period

(Williams *et al*., 2005) and cohabits synergistically with other anaerobe bacteria, like *Fusobacterium necrophorum* (Földi *et al.*, 2006). Most pathogenic bacteria isolated from postpartum cows with uterine diseases are *Escherichia coli, Prevotella* sp. (Sheldon *et al.,* 2006). Also, many studies isolated *Streptococcus* sp., *Staphylococcus* sp., or non-coliform aerobic gram-negative rods (Kaczmarowski *et al.,* 2004; Jadon *et al.,* 2005). To choose a suitable and effective antimicrobial drug to treat postpartum uterine diseases, it is very important to know the sensitivity of the pathogen to antibiotics. Infiltration of endometrium by microorganisms induced inflammatory responses characterized by the secretion of proinflammatory factors such as cytokines and chemokines. This lead to the influx of neutrophils and culminated with bacterial clearance (Hussain, 1989). The endometrium is considered as the first line of immune defense against invading microorganisms, the endometrium is fortified by innate immune capabilities such as Toll-like Receptors (TLRs1 to 10). The endometrium is activated to elicit a number of proinflammatory immune factors such as interleukin 1, 4 and 6 (IL-1, IL-4, and IL-6) which helps to attract and mobilize more neutrophils and monocytes to the endometrium (Davies *et al*., 2008). Thus, assessing these cytokines, chemokines, and acue phase proteins in the blood could relate to the endometrial clinical condition of these animals in our study

Predisposing factors like the physiological status of the cow and accompanied by energy deficiency increases the susceptibility of the dairy cows to microbial and metabolic diseases like endometritis, ketosis, milk fever, displaced abomasum and retained placenta (Duffield, 2000). A complicated relationship is present among factors that affect uterine health and postpartum cows (Kinsel, 1996). Energy balance, particularly negative energy balance, has an immediate effect on reproductive performance in bovine (Grummer *et al.*, 2010; Cardoso *et al.*, 2013). Hammon *et al.* (2006) reported that uterine infection is accompanied with negative energy balance that begins prior to parturition and continues through to the early stage of lactation and reported that cows with acute negative energy balance had reduced neutrophil function. Studies have shown that cows with uterine infection manifest a greater degree of negative energy balance represented by increasing non-esterified fatty acids (NEFA) and beta-hydroxybutyrate (BHBA) (Hammon *et al., 2006)* Ovarian activity is known to play a crucial role in changing the reproductive performance of a cow, first resulting in pregnancy and then calving. Early onset of ovarian activity immediately following calving is important for the onset of timely conception. Uterine diseases at early postpartum period inhibit ovarian granulosa cell activity as well as the growth of dominant follicles (Williams *et al*., 2007) and this causes a suppression in synthesis and release of estradiol resulting in change in follicle lifespan and ovulation (Herath *et al*., 2007).

Newer techniques like uterine cytology have also been used to improve and obtain an accurate diagnosis of endometritis cases in apparently healthy cows. Both cytobrush and the small volume uterine lavage technique can be used to get endometrial cells samples from the uterus (Lincke *et al*., 2007). Cytobrush cytology is considered as the gold standard when compared to other methods (Drillich *et al*.,

2004). The percentages of PMN in the total number of endometrial cells in the uterine sample provide sufficient evidence for subclinical endometritis. Threshold point percentage for the proportion of PMN varied from 5 to 18% (Galvao *et al*., 2009). Ultrasonography is another technique used to reveal the level of accumulation of intrauterine fluid associated with endometritis (Kasimanickam *et al*., 2004). It also provides useful information that facilitates the immediate detection of endometritis. The application of ultrasonography techniques has shown that accumulation of intrauterine fluid is associated with delayed intrauterine involution and increased bacterial growth (Mateus *et al*., 2002).

Problem statement

At present, there is a lack of study about the occurrence of SCE in beef and dairy cows in Selangor state, Malaysia. The treatment failure of uterine infection (possibly due to resistance to antibiotics), and lack of quick and standard diagnostic methods for SCE that depends on PMNs, warranted the need to conduct this study.

Justification

As mentioned above, in Malaysia, there is a paucity of information on SCE in postpartum cows, associated bacterial contaminants and their spectra of sensitivity to antibiotics, diagnosis, as well as effects of SCE on reproductive performance. There is lack of studies about uterine infections like clinical and subclinical endometritis in Serdang, especially in TPU and resumption of ovarian activity during the postpartum period. Our study also focused on investigating the potential risk factors that could affect the occurrence of endometritis and reproductive statuses such as dystocia, parity, body condition score as well as isolation of pathogenic uterine bacteria after 4 weeks postpartum. To choose a suitable and effective antimicrobial drug to treat endometritis cows, it is very important to know the sensitivity of the bacteria to antibiotics. Also, the study tried to look for the interaction between serum proinflammatory cytokines and acute phase proteins and SCE in postpartum cows and the possibility to use them as biomarkers for endometritis in cattle without any clinical signs of other clinical diseases. Our population target of current study involved all calving cows in TPU and some farms in the vicinity.

Research objectives

The main objectives of this study were:

1. To evaluate three different cytological methods to obtain endometrial samples.
2. To correlate the bacteriological findings with vaginal discharges, and endometrial cytology for endometritis detection in postpartum beef cows.
3. To compare the postpartum ovarian activity and energy balance between SCE and healthy cows
4. To measure the interactions between SCE and selected cytokines, and acute phase proteins (APPs)
5. To compare between the use of ultrasonography (US) and endometrial cytology (EC) to diagnosis endometritis in postpartum beef cows.

Research hypotheses

The major research hypotheses can be outlined as follows:

Ho: There is no significant difference among three different endometrial cytological sampling methods and ovarian resumption between SCE and healthy postpartum cows

Ha: There is a significant difference among three different endometrial cytological sampling methods, and the correlation among endometrial cytology (PMN %), vaginal discharges and bacterial findings from postpartum cows. Also, the study proposes significant differences between SCE and healthy cows in ovarian resumption, energy balance, and interaction with selected cytokines and acute phase proteins.

CHAPTER 2

LITERATURE REVIEW

2.1 Postpartum period in cows

Fertility after calving in both dairy and beef cows is considered as the principal economic factor of milk producing and beef farms. Alteration in postpartum period will result in a progressive severe economic loss (Wettemann *et al*., 2003; Patel *et al*., 2006). The endurance of cow in a herd depends largely on the type of programs employed after calving and the benefits associated with their physiological consequences (Bello and Pursley, 2006). The puerperium period can be defined as the phase immediately after parturition until complete uterine involution, which is approximately between 40 to 60 days postpartum in cows. Uterine involution, endometrial regeneration, the resumption of the ovarian activity and getting rid of bacterial contamination are the four important steps that occur concomitantly after calving and expulsion of the placenta (Shedon *et al*., 2008).

2.1.1 Uterine involution

The involution of the uterus, considered as the second important step to resume reproduction in the postpartum period, is characterized by the return of the uterus to the original size and location before the pregnancy (Hafez *et al*., 2000). Involution of the uterus after calving is essential because it helps to prevent uterine infection. On the other hand, the uterine infection has been shown to delay the involution of the uterus during the postpartum period. Besides that, assessment of the uterine and cervical involution can help to distinguish between physiological and pathological clinical conditions of the uterus. Calving abnormalities such as retained fetal membranes (RFM) and dystocia have harmful impacts on the involution of the uterus by increasing the time required to complete during the postpartum period. This creates a medium for invading microorganism to thrive, thus causing uterine infection (Sheldon *et al*., 2006).

The smooth muscles of the uterus also play an important role in getting rid of the content of the uterus and returning the uterus to its normal size before pregnancy (Bajcsy *et al*., 2005). Reduction in the uterine diameter is accompanied at the same time with decreased in the cervical diameter. However, the involution of the cervix is more slowly in comparison with the involution of the uterus (Le Blanc *et al*., 2002). Kasimanickam *et al*. (2004) reported that the cow has large cervix diameter and their involution takes longer time (Table 2.1).

Table 2.1 : The cervical diameter during postpartum period over time in cows.

Cervical diameter	20-33 days postpartum (%)	34-47 days postpartum (%)
<3.5 cm	28.1	57.0
3.5-5 cm	50.5	38.2
>5 cm	21.5	4.8

(Source: Kasimanickam *et al*.,2004)

The significant reduction in uterus size occurs during the first few weeks after parturition. Involution of the uterus is accompanied by a progressive reduction in the diameter of cervix and horns of the uterus. After Parturition, the diameter of the cervix is reduced from 30 cm in length after birth to about 2 cm. The reduction in the size of the cervix facilitates the drainage of lochia across cervix (Wehrend *et al*., 2003). Lochia is normally seen 15-20 days postpartum. Over the course of involution, the colors of lochia gradually change from a red-brown fluid to a more viscous yellow-white material. The diameter of the uterine horn in healthy cows ranges from 3-4 cm by 25-30 days postpartum, while the cervical diameter is less than 5 cm by 40 days postpartum. However, the involution of both the cervix and uterus is not complete until approximately 40-50 days postpartum (Sheldon and Dobson, 2004). Additionally, LeBlanc *et al.* (2002) reported that cows with a cervical diameter of more than 7.5 cm at day 20 to 33 postpartum have reduced pregnancy rate. In contrast, Kasmimantickam *et al*. (2004) reported that there is no relationship between reduction in relative pregnancy rate and cervical diameter. Fonseca *et al*. (1983) noted that there are a number of factors that facilitate uterine evolution and that there is no strong evidence to suggest that delayed involution results to intrauterine diseases.

2.2 Resumption of ovarian activity in cows

The follicular activity is continuous with regular interval 7-10 days during the first six months of the gestation period in cows (Ginther *et al*., 1996). However, this follicular growth is suppressed due to the negative impacts of progesterone and estrogens hormones at a late stage of pregnancy for increasing FSH and stopping follicular waves during the last month before calving (Crowe *et al*., 1998; Forde *et al*., 2011).

Most cows suffer from postpartum anestrus and this transient period is characterized by the absence of sexual activity due to ovarian acyclicity and absence of estrus and decreased of plasma progesterone level to below 0.5 ng/mL (Santos *et al*., 2009a).The reason of postpartum anestrus beyond to delay the recovering of the hypothalamic-pituitary-ovary-uterine axis from the negative feedback of progesterone hormone from previous gestation period (Yavas and Walton, 2000).

Basically, there are two important phases to resume fertility in cattle after parturition and it is represented firstly, by the liberation of the hypothalamus-pituitary axis from the negative feedback for steroid hormones during the late stage of pregnancy and resumption of GnRH, FSH, and LH production. The recovery step is short and the level of LH begins to increase especially in healthy dairy cows (Yavas and Walton, 2000). Also, this step associated with a sharp decreasing of the steroid hormone (progesterone and estrogens) to basal level and gradually increasing of FSH during 7-10 postpartum (Crowe *et al*., 1998). The first follicular waves is enhanced by the first FSH increasing during 7-10 days after calving which develop a dominant follicle (DF) and fate of dominant follicle depended on the amount of estradiol to stimulate the GnRH secretion from hypothalamus and consequently LH pulse which is considered crucial for ovulation (Forde *et al*., 2011). Generally, the first postpartum ovulation in healthy dairy cows occurs approximately by the week two postpartum, while the good body condition beef cows after one month after calving. However, the first postpartum ovulation may be delayed to 75-100 days after calving in weak body condition score (Crowe, 2008).

Pregnancy is considered as one of the most accurate indicators of optimal reproductive performance in large ruminant (Perea and Inskeep, 2008). The resumption of ovarian activity after calving and in early postpartum period is an essential factor to increase the attainment of conception rate in cattle (Yendraliza *et al*., 2011). The onset of ovarian activity and conception in animals is influenced by a myriad of factors such as breed, nutrition, milk yield, suckling time, uterine involution and the season of calving (Pipaon *et al.*, 2002). The presence of a functional corpus luteum and the level of progesterone gives a clear indication of the activity of the ovaries postpartum and successful ovulation (Martin *et al.*, 2010). The transrectal palpation in cows and buffaloes is the most common technique used the method to reveal the activity of the corpus luteum (El-Wishy, 2007). Other methods include detection progesterone in serum and milk (Jazayeri *et al*., 2010) and ultrasonography (Honparkhe *et al*., 2004; Terzano, 2005). Depending on the methods used, the diagnostic value of the different methods is, however, variable (El-Wishy, 2007).

Rectal palpation is a standard technique used for the diagnosis of ovarian structures in cows, but it does not provide accurate and adequate information about the functional activity of the ovaries (Younis *et al*., 1994). Alternatively, analysis of blood and milk progesterone levels is the primary technique that is used to evaluate the functional condition of the corpus luteum (Campanile *et al*., 2010). However, there are a number of factors that may affect the levels of progesterone such as season (Qureshi *et al*., 1999), the presence of bull in the herd (Gokuldas *et al*., 2010) and nutritional condition of the cow (Wongsrikeao *et al*., 1990). Adopting the use of ultrasonographic technique in cow will increase the potential to detect the events occurring on ovaries (Terzano, 2005) and also provides clear images about the ovarian activity in postpartum cows and buffaloes (Yindee *et al*., 2011)

Resumption of ovarian activity at the beginning of the postpartum period is crucial for first timed animal conception. This is because it influences the rate of obtaining the economically acceptable length of the open day period. Essential event critical to the restarting of ovarian function in postpartum period is the emergence of the first follicular wave and selection of the dominant follicle (DF) that may ovulate, become atretic, or convert into a follicular, luteal cyst or non-ovulatory follicle (Sakaguchi *et al*., 2006; Lucy, 2007). Several other factors play a pivotal role in the fate of DFs postpartum which have a close relationship to the metabolic condition of animals. For instance, the level of diet in the postpartum period, energy balance (EB) and parity (Beam and Butler, 1997; Cavestany *et al*., 2009). An in-depth study has been carried out on follicular events and the dynamics of the ovary in cows and heifers during the estrous cycle (Šichtař *et al*., 2010).However, there is no adequate information about the activity of the changing follicle during the early postpartum period, especially in high-production dairy cows.

In relation to the onset of ovarian activity postpartum, a number of studies carried out concentrated only on primary indicators like the dynamics, metabolic and hormonal profiles of the first follicular wave, length of days to the first postpartum ovulation, the onset the activity of the corpus luteum links to uterine involution or to the first insemination (Hommeida *et al*., 2005; Kawashima *et al.*, 2007; Hayashi *et al*., 2008, Galvao *et al*., 2010b). It has been reported that the first follicular growth wave appear immediately after parturition and it is independent of the energy balance (EB) of the cows (Butler, 2003). Savio *et al*. (1990) reported that the selection of the dominant follicle which is more than 9 mm (> 9 mm) occurs at 10 days postpartum. Dominant follicles from the first follicular growth wave ovulate in 30–80% of cows by day 20 (Crowe, 2008) and the period from parturition to the first ovulation seems to be dependent on parity (Tanaka *et al*., 2008).

The successfully ovulated ova develops to corpus luteum (CL) and subsequently increase the production of progesterone hormone. However, luteolysis process after the ovulation period can result in reestablishment of another cycle ovarian activity (Peter *et al*., 2009). Interestingly, Opsomer *et al*. (1998) reported that the onset of the 1st luteal phase postpartum may not be regular, thus, indicating that the follicular growth event may be influenced.

Postpartum anestrus is considered one of the common obstacle that prevents the both Bos *Taurus and Bos indicus* in the tropical area of the achieving one-year calving interval and affects the reproductive performance of these cows. There are many different factors that can affect the length of postpartum anestrus period like BCS, nutrition, breed and environmental stress factors (Ball and Peteres, 2004; Santos *et al*., 2009a).

2.2.1 Factors affecting resumption of ovarian activity

There is a significant relationship between the status of nutrition in cow farms and the reproduction and production of these cows in a tropical area (Montiel and Ahuja, 2005) and BCS is one of the aspects of the nutritional status of the cow farms (Randel, 1990). The imbalance between nutrition intake and the production demands of these cows develops postpartum anestrus especially when these cows depended on grazing to meet their feed requirements (Jolly *et al.*, 1995). Generally, there is a complex relationship between nutrition and other factors like environmental, genetic and management factors about increasing the rate of the prolonged anestrus after calving (Ball and Peteres, 2004; Montiel and Ahuja, 2005). The negative energy balance (NEB) is considered one of the most common reasons that have adverse effect on the ovarian resumption after calving in dairy and suckled beef cows, and supply these cows with dietary energy may reduce the length of postpartum period and keep these cows in optimal BCS (Ball and Peteres, 2004; Crowe, 2008).

The rate of consumption of dry matter intake has a significant relationship with energy balance, duration, and severity of NEB in postpartum cows is related to intake of dry matter and the status of BCS after calving (Bulter, 2000). The NEB after calving develop prolong postpartum anestrus by inhibition the secretion of LH, reduce the sensitivity of ovaries to stimulation by LH and also reduce serum glucose and IGF which very essential to the ovarian resumption of the postpartum cows (Bulter, 2000).

The onset of ovarian resumption, follicular growth, ovulation, and estrus behavior are suppressed in both dairy and beef suckled cows (Ball and Peteres, 2004). The mechanism of the effect of suckling on ovarian activity is not clear but some of the studies reported the suppressive impact of sucking on LH secretion from the anterior part of the pituitary gland by inhibition of hypothalamic GnRH (Montiel and Ahuja, 2005). However, the previous study by Yavas and Walton (2000) concluded in their study that there is less effect of sucking after day 45 postpartum on LH pulse and ovarian activity.

The debate about the impact of milk yield on ovarian resumption and follicular growth is still continuous; some of the studies reported the adverse effects of high production milk in cows on the follicular growth and delay the resumption of ovarian cyclicity of these cows after calving (Santos *et al.*, 2009a). Maybe the high milk production after calving develops NEB in these cows and consequently delay of ovarian resumption. However, another previous study did not find clear evident between milk yield and ovarian function after parturition (Ball and Peteres, 2004). A previous study by Lucy *et al.* (2001) found the minor impact of milk production on reproductive performance compared with other factors.

The direct studies about the effect of the breed on the resumption of ovarian activity and length of postpartum anestrus are not clear and limited. There is no consensus for the many studies about the effect of breed on reproductive performance in cow farms. However, one study was reported the high sensitivity of LH to response to GnRH in Brahman (*Bos indicus*) compared with Angus cows (*Bos taurus*) (Stahringer et al., 1994). However, another report by Randel (1976) revealed the higher plasma LH level in *Herford* cows (*Bos taurus*) compared with *Brahman* (*Bos indicus*). A new study by Yimer (2011) reported the decreasing of a number of cows that had ovarian dysfunction in Kedah-Kelantan (K.K) cows compared with other beef cows like Brangus by using serum progesterone levels as an indicator of ovarian activity in UPM cows farm, Malaysia.

The heat stress and humidity are the main factors of environmental effects which have a strong adverse impact on fertility of cows by decreasing conception rate and increased embryo mortality at both Bos Taurus and Bos indicus (Salam *et al*., 2006). The prolonged anestrus is considered one of the main problems that Bos Taurus suffered from it at the tropical area (Ball and Peteres, 2004).

A previous study that was conducted in Tunisia concluded the adverse impacts of temperature stress on the reproductive performance of dairy cows by affecting the days open and increased calving interval for the cows that exposure to heat stress (Salam *et al*., 2006). Another study by Wilson *et al*. (1998) reported the inhibition effect of heat stress on follicular growth and the delaying of luteolysis of corpus luteum in heifers.

2.3 Metabolic and production pressure on uterine immunity

The majority of the health problems in cows herd usually begins during periods that precedes calving. This is believed to be due to the physiological imbalances as a result of the adaptations to milk production (Ingvartsen, 2006). For instance, the setting up of uterine infection takes place around the same time as peak milk production and the incidence of the uterine disease is greater in high-lactation cows. Crowe and Williams (2012) reported that about 73.3% cows yielding more than the median value of 35 kg milk/day are prone to endometritis and metritis compared with the 45.2% of cows that produce less than 35 kg milk/day. This change is believed to be due to the major effect of negative energy balance (NEB), with cows that yield larger amounts of milk having a deficiency of energy after birth.

The relationship between infection, metabolic diseases, and reproductive performance are comprehensively reviewed. On a review by LeBlanc (2012), NEB plays a vital role in upsetting the immune capabilities of the uterus and increases the chances of reproductive tract inflammation and postpartum disease. Additionally, Hammon *et al*. (2006) reported that uterine infection that occurred as a result of negative energy balance usually begins prior to birth and continued through the early

stages of lactation. The authors further opined that cows with acute negative energy balance had reduced neutrophilic function. Besides that, severe negative energy balance is usually accompanied by an elevated expression of inflammatory factors in the endometrium 2 weeks postpartum (Wathes *et al*. 2009). Thus, suggesting that the negative energy balance disrupted immune function, which may affect the predispose the dairy cows to uterine infection and subsequently reduce their ability to overcome post-partum uterine infection.

Interestingly, a greater degree of negative energy balance usually by increased non-esterified fatty acids (NEFA) and β-Hydroxybutyric acid (BHBA) during closed calving is seen in cows with intrauterine infection (Galvao *et al*., 2010a). Burke *et al*. (2010) however, reported that cows suffering from endometritis had the same profiles of glucose and NEFA like healthy animals, and neither NEFA or BHBA during onset lactation was registered to have been accompanied with metritis or subclinical endometritis (Valergakis *et al*. 2011; Senosy *et al*. 2012). This finding indicated that energy condition, as assessed by metabolite levels, is not a risk factor for uterine infection during the postpartum period. The reason for the disparity between these findings is not clear. Metabolic condition and NEB are the results of mixed factors which includes body score, milk production, dry matter intake and parity, so it may be possible that differences in any of these factors contributing to NEB may affect infection outcome.

It was observed that cows that are obese are 3.6 times more likely to show a purulent vaginal discharge 14 days postpartum after calving (Williams *et al*., 2009). Additionally, it was also observed that progressiveness in parity is accompanied with higher milk production and an elevated risk of endometritis, increased culling rate and reproductive impairment (Lee and Kim, 2006). In a recent study, it was observed that cows that are suffering from clinical endometritis have a relatively higher milk production capability than their healthy herd mates (Giuliodori *et al*., 2013). Interestingly, even though there is sufficient evidence to suggest that NEB can influence immune activity in the post-partum cows and thus increasing the risk of uterine infection, a lot of contrasting data abounds which makes it difficult to successfully draw a conclusion on the effect of NEB on uterine infection during the postpartum period.

2.4 Uterine bacterial contamination and infection

Microbial contamination of the uterus immediately after calving forms the basis for the high rate of inflammatory responses that usually occur postpartum. There is a clear distinction between uterine disease and uterine contamination. For instance, at birth, it is a normal phenomenon to be contaminated with a wide range of microbial agent without showing any clinical signs of disease. However, following immunosuppression, the microbial agent can colonize the uterine endometrium, infiltrate the mucosa and produce toxins that lead to uterine infection (Janeway *et al*.,

2001). These microbial contaminants in most cases disrupt the uterine function and subsequently reproductive failure (Sheldon and Dobson, 2004).

Studies have shown that bacterial organism can survive in samples collected from the endometrium two weeks after birth. Even though cows have the ability to wade through this microbial contaminants, it was observed that in about 10-17% positive bacterial samples still abounds many weeks after birth (LeBlanc *et al*., 2002). The presence of this pathogens and their toxins elicits inflammatory responses, impairment of uterine involution, uterine tissue damage, disorders of the ovaries, ovarian failure and reduce the survivability of the embryo (Sheldon *et al*., 2004).The success of uterine infection is based on the bacterial load and immune capabilities of the animal. This is because the number of microbial agents in the uterus of postpartum animals may be significant enough to overshadow the uterine defense mechanism and even threaten the life of the animal (Sheldon and Dobson, 2004). The survivability of the uterine microflora is partly dependent upon the endocrine system. For instance, progesterone is known to limit the function of the uterine defense mechanisms. Additionally, the establishment of the first corpus luteum followed calving and secretion of progesterone hormone in most cases precedes the onset of uterine infection (Lewis, 2004). Studies have also shown that the invasion of the uterus postpartum by a wide variety of pathogenic bacteria did not produce uterine infection until the blood progesterone levels were increased (Del Vecchio, 1992). In addition to that, Heuwieser *et al*. (2000) also reported that the contamination of the uterus postpartum is strongly influenced by the amount of progesterone produced by both the adrenal gland and the corpus luteum. There is a need for further assessment of the relationship between uterine microbial flora, ovarian activity and inflammatory processes in the uterus, although, uterine infection is accompanied with ovarian cysts and anestrous cases (Opsomer *et al*., 2000).

2.5 Types of uterine infection

The uterus is exposed to several types of microbial infection after parturition and this causes a severe economic loss in cattle production (Sheldon *et al*., 2006; Evans and Walsh, 2011). Bacterial infection of uterus leads to uterine diseases and decreased the reproductive performance of cows by disrupting the function of ovaries and uterus of cows in the postpartum period. The immune system of the body plays an essential role to recognizes the different pathogenic agents and rid the genital system of these microbial agents (O'Neill, 2008; Yasui *et al*., 2014). Although, contamination of uterus by several types of non-specific bacteria is eliminated during involution process of the uterus. There are approximately more than 25% cows whose immune system cannot effectively remove this pathogenic organism thus, resulting to uterine infection like metritis, endometritis and make a substantial number of these as cows as repeat breeder (Singh *et al*., 2008).

2.5.1 Metritis

Metritis is defined as the inflammatory response of uterus due to infection by different pathogenic microbial agents with a systemic reaction such as increased body temperature of more than 39.5 ℃, heart rate, respiratory rate, loss of appetite, and decreased milk production. Metritis is associated with watery red-brown foul smelling discharges of the uterus. The infection occurs during the first three weeks after calving and is accompanied with cases of dystocia, retained placenta, stillbirth, twinning and delayed of uterine involution (Drillich *et al*., 2001; Sheldon *et al*., 2006). Studies have shown that more than 40% of cows one week after calving are exposed to uterine diseases and incidence of the cases varied between 36-50% in cows (Zwald *et al*., 2004). Additionally, about 20% of cows are suffering from systemic signs of metritis (Benzaquen *et al*., 2007). Furthermore, more than 15% of cases of metritis in cows develops to clinical endometritis three weeks after calving while 30% to subclinical endometritis(Gilbert *et al*., 2005; Sheldon *et al*., 2006).

Retained fetal membranes are considered as one of the most common predisposing factors of metritis in cows (Smith and Risco, 2002). For example, about 20-50 % of cows are suffering from metritis. Additionally, Huzzey *et al*. (2007) demonstrated a relationship between dietary intake and the development of metritis during the postpartum period. The study showed that cows with daily feed intake of less than 5 kg are more likely to have metritis than healthy cows fed ad libitum.

There is a lack of precise information about the efficiency of using treatment for metritis or safeguarded programs to avoid developing severe of metritis and any disorder related to infection like a displaced abomasums or to get satisfied results of reproductive performance of postpartum cows. For instance, giving ceftiofur HCl to a cow that suffers from retained fetal membranes regardless of whether these cows have a fever or not will produce a significant result (Risco and Hernandez, 2003). In trying early detection of metritis, it is important to check the normal involution of the uterus during the postpartum period and to differentiate between lochia and abnormal uterine discharges as a result of uterine infection. Additionally, look for indices like the posture of cows, appetite, and milk yielding of postpartum cows (Sheldon *et al*., 2004).

2.5.2 Pyometra

It is defined as the accumulation of a large amount of pus or mucopurulent material in the lumen of the uterus due to the activity of pathogenic bacteria which lead to the distension and enlargement of the size of the uterus with active corpus luteum on one of the ovaries. This condition usually occurs after first ovulation in postpartum cows before the clearance of contamination of uterine lumen from pathogenic bacteria (Foldi *et al*., 2006; Chapwanya, 2008). Due to incomplete closure of the cervix, some of the discharges passed through the cervix into the vagina and seemed like clinical signs of clinical endometritis, so it should be differentiated. The incidence of

pyometra is less than clinical endometritis, and it forms 5% of uterine disease when compared with clinical endometritis which is more common in the postpartum period. Fortunately, using luteolytic drug such as PGF2α in both cases were effective as a treatment. Pyometra case could be diagnosed in cattle by using ultrasound technique and appeared by the existence of corpus luteum on an ovary and echo density fluid in the lumen of the uterus (Sheldon *et al*., 2006).

2.5.3 Endometritis

Endometritis is one of the major problems affecting dairy and beef cows immediately after birth thus, leading to impaired reproductive function (Wettemann *et al*., 2003; Sheldon *et al*., 2006). It is defined as the inflammation of the superficial layer of the endometrium and not deeper than the stratum spongiosum (Bondurant, 1999). Endometritis that occurs immediately after birth has a passive effect on the reproductive performance of the cow. This is because it increases the level of service per conception, calving to the first period of insemination and calving to period of conception (Heuwieser *et al*., 2000). Additionally, diminishes the rate of conception and impair the chance of pregnancy (LeBlanc *et al*., 2002). Gilbert *et al*. (1998), reported that prevalence of endometritis in herds of cows ranges from 7.8 to 61.6%. In Croatia, Zobel (2013) reported that 23.07% of a total of 1300 cows sampled had endometritis. Additionally, clinical and subclinical endometritis were observed in 15.31% and 7.77% of cows respectively. In Japan, Gautam *et al*. (2009) reported 67.8%, 40.5% and 14.4%, incidence of metritis in two fields during early, intermediate and late postpartum period. Losses due to different types of uterine infection were estimated to be about 285 USD per lactation (Guard, 1994). The early and accurate detection of endometritis is stalled due to the lack of agreement on a universally acceptable definition of the case and diagnostic method (Gilbert *et al*., 2005). This is further compounded by the fact that a large proportion of postpartum cows manifest different signs of uterine inflammation that accompany normal involution. It is there for pertinent for veterinarians to be able to accurately and precisely identify early onset of endometrial infection in order to institute appropriate treatment (Sheldon *et al*., 2006).

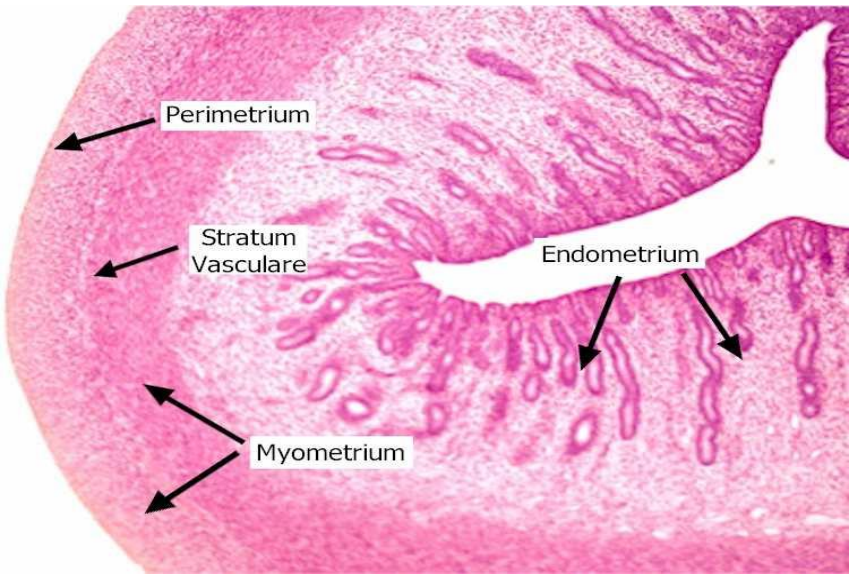

Figure 2.1 : Histological section of the cow's uterus.

2.5.3.1 Clinical endometritis

Clinical endometritis in cattle is known as inflammation and infection of the endometrium of the uterus more than two to three weeks after birth without systemic signs of illness. Infected cows show signs of purulent–like discharge on the tail and vulva, purulent (21 days after calving) or mucopurulent (26 days postpartum) discharge from the vagina. The diameter of cervix becomes greater than 7.5 cm after 20 days post calving and stinky materials (McDougall *et al*., 2011). Alteration with a step up time after calving and the size of the cervix is the standard method of diagnosis (LeBlanc *et al*., 2002). The incidence of clinical endometritis is seen in about 10% to 27% in cow herds (LeBlanc *et al*., 2002; Dubuc *et al*., 2011). Clinical and subclinical endometritis constituted one of the major causes of severe economic losses in cows due to a reduction in milk production, decreased fertility and increased culling rate (Pleticha and Heuwieser 2009), hence, the need to prioritise the condition. Clinical endometritis is light to diagnosis, and it is considered as a usual practice for each herd to be routinely examined with the transrectal palpation technique (Burke *et al*., 2010); hence, cows that suffer from clinical endometritis will most likely be diagnosed and handled. On the other hand, subclinical endometritis (SCE) is not easing to diagnose and can lead to a high probability of reduction and impairment of reproductive performance of the infected cows. This is one of the reasons that the recurrent search's focus would be mainly on SCE.

2.5.3.2 Subclinical endometritis

Subclinical endometritis (SCE) is known as endometrial inflammation of the uterus without the accumulation of mucopurulent material in the vagina (Sheldon *et al*., 2006) and cows do not show systemic symptoms of sickness or clinical disease

(Barlund *et al*., 2008). However, inflammation of the upper layer uterus characterized by the infiltration of PMN cells evident (Sheldon *et al*., 2009). Subclinical endometritis is also referred to as 'cytological endometritis' (Gilbert *et al*., 2005; Dubuc *et al*., 2010). Dubuc *et al*. (2010) Described cytological endometritis as "an elevated ratio of PMN in endometrial cytology samples obtained by using cytobrush or low-volume uterine lavage endometrial". Subclinical endometritis is seen in a relatively large number of cows. For example, an incidence rate of about 50% has been reported in cows seven weeks post calling in high yield dairy herds in North America (Sheldon *et al*., 2006). Additionally, Carneiro *et al*. (2014) reported that the prevalence of subclinical endometritis in the field was about 26 % and the condition was not influenced by season of birth, the existence of corpus luteum, milking parity and days in milk (DIM). Gilbert *et al*. (2005), also reported that the incidence of cytologically-identified endometritis in five commercial dairy farms located in central New York was initially 53%; the rate however changed from 37 to 74%. Dairy cows that suffered from clinical and subclinical endometritis are associated with reduced fertility (Kasimanickam *et al*., 2004; Hammon *et al*., 2004). The primary effect of subclinical inflammation appeared to be impaired first-insemination pregnancy rate, which conducted to elongated days open of these cows. Cows with subclinical endometritis were at a greater risk of not getting pregnant which might lead to the culling of the affected cows in the herd (Gilbert *et al*., 2005). This is because proinflammatory and cytokines mediators that associated with inflammation have a damaging effect on embryo survival (Hill and Gilbert, 2008). As previously defined, SCE is the inflammation of the superficial layer of the uterus (endometrium) (Sheldon *et al*., 2006; LeBlanc, 2008). The inflammatory reaction is part of the body's defense mechanism to wade of infection (Serhan *et al*., 2010). Some of the processes that occur during an inflammatory response includes recognition of an wound or a foreign particle (antigen), Stimulation of endothelial cells to secrete pro-inflammatory cytokines and chemokines, infiltration of polymorphonuclear cells (in particular neutrophils) to the site of the injury/antigen via chemotaxis in response to the cytokines and chemokines, stimulation the release of acute-phase proteins such as haptoglobin(Hp), and serum amyloid A (SAA) from the liver in response to proinflammatory cytokines, and phagocytosis of foreign invading particles by PMN (Tothova *et al.,* 2008; Sheldon *et al*., 2009; Serhan *et al*., 2010).

Inflammation is induced by prostaglandins that stimulate the release of cytokines (Bos *et al*. 2004). Prostaglandins are bioactive lipids that are derived from the conversion of arachidonic acid by the cyclooxygenase enzyme (COX) into PGH2, which is then further metabolized into various prostaglandins such as series F, E, I, and D prostaglandins (Drillich *et al*., 2007). The COX-1 enzyme is constitutively expressed in cells; COX-2 is not, but its expression is induced by some cytokines, endotoxins, and inflammation (Drillich *et al*., 2007). In the case of bacterial infection, an inflammatory response is elicited by bacteria infecting the uterine endometrium (Cheong *et al*., 2011).

Due to the absence of definitive clinical signs, the most convenient and powerful diagnostic method is still a matter of debate. Although endometrial biopsy may be considered as the ideal method of endometritis diagnosis, the procedure is invasive, expensive, and time-consuming (Herath *et al*., 2009). Furthermore, it has been shown that an endometrial biopsy has a potential of impairing future fertility (Etherington *et al*., 1994). Currently, the standard technique used for the diagnosis of SCE is the quantification of the polymorphonuclear cell (PMN) among uterine cells by the cytobrush technique (Kasimanickam. *et al*., 2004). The presence of more than 18% polymorphonuclear (PMN) cells in uterine cytology samples collected 21–33 days postpartum, or greater than 10% PMNs in samples taken at days 34–47 defines the diagnosis SCE (Kasimanickam. *et al.*, 2004, Sheldon *et al*., 2006). A change in cellular structure of cytobrush samples of cows that is suffering from SCE is also reversed by a great messenger RNA (mRNA) expression of lipocalin-type prostaglandin D synthase, IL1A, IL1RN, IL6, TNF, and CXCL8 in endometrium cells obtained by cytobrush method compared with healthy cows (Ghasemi *et al*., 2012).

After microbial contamination, numerous proinflammatory cytokines and chemokines, like interleukin-6 (IL-6), tumor necrosis factor (TNF-α) and IL-8, are secreted into the uterus (Fischer *et al.*, 2010). IL-8 is an effectual chemotactic factor conscripting PMNs to the location of inflammation (Mette *et al*., 2010), IL-8 and TNF-α levels were increased in the tissue of uterus within a few hours of microbial influx into the equine uterus. Increased *il6* gene expression (proinflammatory cytokine gene expression) has been revealed in uterine biopsies from both mares and cows susceptible to showing subclinical endometritis (Ishikawa *et al*., 2004; Fumuso *et al*., 2007). Gabler *et al*. (2009) were the first to report that endometrial cells sampled with a cytobrush can be utilized in order to obtain proinflammatory gene expression. This approach gives room for the utilization of more invasive endometrial specimens. A subsequent finding by Fischer *et al.* (2010) reported elevated il8 and *TNF* gene expression (proinflammatory cytokine gene expression) in the bovine uterus that suffered from clinical and subclinical endometritis, thus proposing that a potential detection threshold can exist. One of the objectives of our study will be about detecting proinflammatory cytokines and chemokines TNF-α IL-6 and IL-8 and relationship with PMN cells in postpartum cows

Less invasive techniques for the assessment and detection of SCE have included using analysis of metabolic blood factors, antibodies, inflammatory blood mediators, and the cellular structure of blood: In cows that are suffered from SCE Heidarpour *et al*. (2012) reported a higher serum level of haptoglobin, b-hydroxybutyrate (BHB), and entire sialic acid compared with healthy cows. Serum level of nonesterified fatty acids (NEFAs), BHB, bilirubin, and urea at week -1(before birth), at wk +1, and at wk +5 (after calving) about birth, were not acceptable for prediction of SCE (Kaufmann *et al*., 2010). Levels of nitric oxide, an inflammatory mediator, in both serum and uterine discharges, were greater in infected cows with subclinical and clinical endometritis when compared with healthy cows (Li D *et al*., 2010). Due to the factors stress condition before and after birth and metabolic disorders during the

postpartum stage, the effect of increasing milk yield and negative energy balance (NEB), a greater rate of animals are susceptible to low immune response and to suffer from SCE. The detection of BHB and NEFAs in serum during the course of our study is necessary to in order assess the levels of these metabolic materials in suspected SCE cows and their relationship with body scores of these cows.

2.6 Pathogenesis of bacterial uterine infection

After calving, the uterus of animals is contaminated with several types of pathogenic bacteria and more than 80% of the postpartum cows suffer from uterine contamination with *Escherichia coli, A. pyogenes, Staphylococcus sp, Clostridium sp, Streptococcus sp, Fusobacterium sp, Pasteurella multocida* and *Bacteroides sp* by 3-4 days after calving. The number of these pathogenic bacteria, however, decreased substantially in healthy cows (Sheldon *et al*., 2004). Studies have shown that uterine contamination with *Escherichia coli* in the first week after calving and *A. pyrogens* in the subsequent week is accompanied by a uterine infection like endometritis (Williams *et al*., 2007). The existence of *A. pyogenes* after three weeks of calving is accompanied by clear signs of mucopurulent discharges from genital tract (Williams *et al*., 2005). The presence of pathogenic microbes in the uterus causes inflammatory responses, histological lesions of the endometrium, impairment of uterine involution, ovarian disorder, ovulation failure and disorder embryo survival (Sheldon *et al*., 2004). Some studies showed that cows with huge uterine contamination of pathogenic bacteria after calving have a small size of dominant follicles on the ovarian waves and develop small corpus luteum and decreased the level of peripheral blood progesterone (Williams *et al*., 2007).

The effect of uterine infection on the function of ovaries is mediated by a different pathway on the ovary, pituitary, and hypothalamus. Cows that suffered from uterine infection have decreased levels of plasma estradiol due to small sizes of dominant follicles. Also the uterine infection may have effects on the pituitary-hypothalamus pathway which are represented by decrease in the mode of action of oestradiol-induced LH surge to ovulation process and this is explained due to presence of LPS (lipid polysaccharide) and proinflammatory cytokines like tumour necrosis factor (TNF) and interleukin (IL)-1 that block GnRH hormone secretion and decreased the responsiveness of pituitary gland to GnRH pulses. However, there is no clear evidence to suggest decreased plasma FSH level during uterine infection (Williams *et al*., 2001; Sheldon *et al*., 2002).

2.7 Antibiotic sensitivity test

Most pathogenic bacteria isolated from postpartum cows with uterine diseases are *Escherichia coli, Prevotella* spp. (Sheldon *et al.,* 2006). Also, many studies isolated *Streptococcus* sp. *Staphylococcus* sp., or non-coliform aerobic gram-negative rods (Kaczmarowski *et al.,* 2004; Jadon *et al.,* 2005). To choose a suitable and effective antimicrobial drug to treat postpartum uterine diseases, it is very important to know

the sensitivity of the pathogen bacteria to antibiotics. Antibiotic resistance has emerged as an essential serious problem in veterinary science and human medicine (Takamtha *et al.*, 2013).

The isolates were tested for their sensitivity to various chemotherapeutic agents by disc diffusion method (Jorgensen and Ferraro, 2009). The test was performed using Mueller Hinton agar. The zones of growth inhibition around each of the antibiotic discs are measured to the nearest millimeter. The diameter of the zone is related to the susceptibility of the isolate and to the diffusion rate of the drug through the agar medium (Jorgensen and Ferraro, 2009).

2.8 The bovine immune response

After birth, contamination of the uterine lumen with a wide range of microbial agents influence the majority of bovine herds (Williams *et al*., 2007), and while a large proportion of cows have the ability to clear these pathogenic microbial agents efficiently, about 25– 30% of these animals have a continuous inflammatory reaction that resulted to impaired the fertility (Gilbert *et al*., 2005; Sheldon *et al*., 2009). The endometrium is considered as one of the primary fronts of the uterine mechanism of defense against the pathogenic microbial agent that ascend the uterus post calving (Herath *et al*., 2009). Additionally, it helps to provide a physical barrier against disease, the endometrium has an essential role in innate uterine immunity, and this has been clearly identified in many of reviews (Sheldon *et al*., 2009; Turner *et al*., 2012).

Body's defense that is represented by the humoral and cellular responses of local non-particular and particular immunity plays an essential role in clearing the uterine infection. The consequence of endometritis in cows is accompanied with very complicated processes including the detection of microbial element by innate uterine immune cells such as Toll-like receptors, the secretion of the tumor necrosis factor-α (TNF-α) and interleukins (IL)), and the influx of neutrophils (Herath *et al*. 2009; Turner *et al* . 2012). Proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 and chemokines (IL-8) hasten neutrophilic and monocytic invasion of the endometrium through chemoattraction that facilitates elevated phagocytosis (Singh *et al*., 2008). The cow's uterine response to microbial infection is fractionally mediated by the realization of pathogenic agent accompanied molecular modes (PAMPs) by the Toll-like receptor (TLR) pathway. Cells of endometrium respond to microbial LPS (lipopolysaccharides) by TLR4 and consequently secrete different types of proinflammatory cytokines and chemokines (Sheldon *et al*., 2010; Cronin *et al*., 2012). Besides that, responses to PAMPs that stick to TLR1, TLR2, TLR5, and TLR6 have also been registered in the endometrium of cows during the gestation period and in the endometrial cell of humans (Silva *et al*., 2012). These processes in most cows develop to eliminate microbial contamination and enhance epithelial healing after which the inflammatory reaction has decreased or is switched off. Cows that suffer from inflammatory uterine infection, the span, and range of reaction are

not controlled validly with damaging impacts on ovarian and uterine function (LeBlanc, 2012).

A significant endometrial pro-inflammatory reaction in the first-week post-calving, as manifested by the elevated endometrial formulation of TLR4 and a higher IL-1/IL-10 rate, is accompanied with existing endometritis and subfertility (Herath *et al*., 2009). Besides that, the local immune reaction in the uterus, unnatural peripheral immune activity before and after birth predisposes animals to uterine infection (Lewis, 1997). According to Tothova *et al*. (2008), proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 are also effective stimulators for the outputting of acute phase proteins (APPs), like serum amyloid an (SAA), haptoglobin(Hp) and acid glycoprotein, The function of these proteins is to aid in clearing of pathogenic agents, e.g., during the customization of other immune proteins or activation of phagocytosis. The defense function of these proteins against the harmful effects of enzymes constituted during the inflammatory reaction that may develop to organ hurt. Acute phase proteins are secreted by the liver, and their levels in the blood of animals elevated over the early few weeks after parturition, in response to uterine diseases caused by pathogenic microbial agents. In spite of the possibility of synthesis of these proteins (APPs) outside the liver, their existence in cells of the endometrium of cows was not reported *in vitro* (Davies *et al*., 2008). However, studies carried out by Chapwanya *et al*. (2013) proposed that the secretion of serum amyloid A by endometrium of cows is possible. Endometritis is also accompanied by a reduction of neutrophilic activity that begins before birth (Hammon *et al*., 2006). It was presumed that individual differing in the immune protocol of gestation period and calving and the reversal of these shifts in the post-calving period are essential drivers of susceptibility to uterine diseases (Hansen, 2013). The capacity to detect cows with sub-perfect immune function before or soon after birth will provide an opportunity to prevent the incidence of uterine infection and improve reproductive performance.

2.9 Diagnosis of endometritis

Subclinical endometritis is detected late compared to other types of uterine infection due to the absence of clear clinical signs associated with the reproductive system, often when insemination becomes non-efficient. Although many studies have been carried out, the effectuation of the new diagnostic technique and the use of varied curative methods, endometritis in cows herds continues to be a significant economic problem worldwide due to high losses associated with decreased ratio of artificial insemination and the needfulness to cull cows in the herd (LeBlanc, 2008; Galvao, 2012).

2.9.1 Transrectal palpation

Although, in reality, the use rectal palpation of the uterus is considered as one of the most common practically available methods for diagnosis of endometritis especially

clinical type. Several studies have however reported that methods cannot accurately diagnose cows suffering from endometritis and other impairment of the reproductive tract (Runciman *et al*., 2008).

2.9.2 Vaginoscopy

This method is considered as one of the most common and rapid techniques used to assess the accumulation of uterine and vaginal discharge in the vagina with the aid of a vaginoscope (LeBlanc *et al*., 2002; Sheldon *et al*., 2006). The 4-point scoring system (0 = clear mucus, 1 = mucus containing flecks of pus, 2 = discharge containing less than 50% pus, 3 = release containing more than 50% pus) used to categorize vaginal discharges was invented by Williams *et al*. (2005) and report has shown that it is been utilized in later studies (Sheldon *et al*., 2006; Kaufmann *et al*., 2010). An essential obstacle in ratifying many diagnostic techniques and prescribing test characteristics is a deficiency of a gold standard to achieve infection of the uterus (Drillich *et al*., 2007). Besides that, the existence of mucopurulent discharges in the vagina may not provide sufficient evidence to endometrial infection (Dubuc *et. al*., 2010). Cytological screening is one of the most accurate methods for the detection of CE and it enables the distinction between CE and other types of inflammation of genital tract like cervicitis and vaginitis (Sheldon *et al*., 2006).

Vaginoscope is a simple instrument that is used to assess both vagina and cervix in post-calving cows. It is recommended as a standard inspection tool to be employed especially if transrectal palpation method is the only other diagnostic modality that is available in cows herds (LeBlanc *et al*., 2002). Barlund *et al*. (2008) reported that vaginoscopy as a technique is less sensitivity when compared to endometrial cytobrush cytology for the detection the of CE and SCE in postpartum cows, but recommend using this method to help in detection of CE in cows that are four weeks after calving. Similarly, Drillich *et al*. (2004) reported that the vaginoscopy technique has a sensitivity of about 12.3% and 90.2% specificity when compared to cytobrush cytology results as a reference. The results of the assessment of vaginoscopy to reveal cases of clinical endometritis in cows showed that the technique is not optimal but can be regarded a sensible scales of discharges that accumulate in the vagina and is a practical instrument that differentiates between infected cows and healthy cows in the herd.(Leutert *et al*., 2012).

2.9.3 Endometrial cytology

Uterine cytology involves calculation of cells obtained from the uterus and has a standard when are compared with other diagnostic methods for the detection endometritis in bovine (Barlund *et al.*, 2008; Dubuc *et al*., 2010). Subclinical endometritis is detected by counting the rate of PMN that infiltrate the uterine tissue by cytology sample (Sheldon *et al*., 2009). There are two cytological methods mostly employed to detection SCE (Kasimanickam *et al.*, 2005) and these include the cytobrush technique and the uterine flushing/lavage technique. Briefl**y**, the cytobrush

technique involves a cytobrush that is inserted through the cervix and softly circuited against the endometrium; the cellular contents are then scrolled on a slide for microscopic assessment to count the number of cells that are PMN within the uterine sample (Kasimanickam *et al*. 2004; Barlund *et al.,* 2008). Uterine lavage involves flushing of the uterine lumen with a small amount of isotonic solution, which is recovered and evaluated by microscopy (Gilbert *et al*., 2005). In spite of the benefits of the uterine lavage, Kasimanickam *et al*. (2005) reported that the cytobrush technique is a more ordered and reliable than uterine flushing, uterine lavage has about a 17% unsuccessful rate to getting samples due to a decreased count of PMN recovered and elevated misshaping of these cells. For this cause, the cytobrush method will be employed in this study.

In trying to clarify this finding, a sample from randomly selected slides organized using endometrial material was tested side by side, the result showed that cytobrush slides were more distinct due to the presence of more PMNs, mucus, endometrial debris and another endometrial cell than cytological flushing technique. Reasonable cause for the decreased in the definition is that the flushing procedure may negatively influence the integrity of the endometrial cells. Also, the pH of available saline used was 5.7 and may be changed ranging from 4.7 to 7.0. The second reason for cellular endometrial damage could have occurred during the centrifugation operation. Even though 600 x g is recognized to be the most acceptable speed limit for cells, it may still affect the integrity of small amount of cells when the ti,e exceed 15 minutes (Barlund *et al.,* 2008)

A diagnostic threshold value for the percentage of PMN should be applied to check whether a cow suffers from SCE or not. The percentage of PMN threshold is utilized to determine cows that suffer from SCE. This threshold value varies based on the time post-calving that the endometrial cytology sample was collected and the results being investigated. In fact, the PMN value threshold depended on reduction as time post-calving increases (Kasimanickam *et al*., 2005; Gabler *et al*., 2010). This is due to the reduction of uterine infection as time post-calving increase as a result of the advancement process and completion of the involution of the uterus (Gilbert *et al*., 2005).

Researchers have applied several approaches to find out what PMN threshold depended on, like using a PMN % that was accompanied with an adverse effect on optimal fertility. Kasimanickam *et al*. (2004) utilized the first approach. The authors employed the use of different selection analysis to depend on the lowest value of PMN % which was accompanied with a passive impact on optimal fertility. Additionally, it was reported that cows have more than 18% PMN at 20 to 33 days postpartum and more than 10% PMN at 34to 47 days post-calving SCE cows. Burke *et al*. (2010) employed the use of another approach that the PMN % threshold depended on. The author classified the animals into quartiles(≤1%, 2–3%, 4–6%, and >6%); the highest PMN % value quartile (> 6% PMN at day 42 postpartum) define cows that suffer from SCE. Both procedures have advantages and disadvantages.

The first method is better in that it determines its PMN % threshold based on touchable results. For example, pregnant or not in a particular time followed the beginning of the coupling period. There are several agents other than PMN %, like time from birth to coupling and mating management (Barlund *et al*., 2008; LeBlanc, 2008). The benefit of the second method is that it skips the rounded discussion about depending on a threshold value for optimal fertility of these cows and then using this to evaluate the parameters of reproductive performance of these cows (McDougall *et al*., 2011), but there is the likelihood that the higher quartile PMN % may not be accompanied with the best fertility. In general, PMN cells are depended on the detection of SCE due to fact that it as an optimal guide of the level and the severity of inflammation associated with uterine infection. It also considers overriding the inflammatory cells that are present in the fluid that accumulates in the uterine lumen (Barlund *et al*., 2008). The PMN cells are a portion of the bodies defense system that protects the body against invading pathogenic microbial (Paape *et al*., 2002). Therefore, PMN cells infiltrate into the uterine tissue when any microbial contamination develop an infection (Kasimanickam *et al*., 2004).

2.9.4 Ultrasonography

Ultrasonography considered as one of the most common diagnostic tools that have recently been employed in veterinary medicine. Most of the studies have been concentrated on the existence, volume and complexion of fluid that accumulate in uterine luminal. Mateus *et al*. (2002) studied post calving cows by using transrectal ultrasonography and found out that the volume of intrauterine fluid was significantly accompanied with a reduction in the duration of uterine involution, and there is a strong relationship between the grade and volume of intrauterine fluid and pathogenic microbial growth. It appears plausible that any inflammatory reaction within the endometrium may develop a different degree of endometrium tissue thickness. A trial was made to assess using transrectal ultrasonographic to measure the thickness of endometrium as an indicator to cows that suffer from endometritis. Barlund *et al*. (2008), reported the use of endometrial thickness measurements scale were less precise than endometrial cytobrush with a sensitivity of 3.9% and a specificity of 89.2% when compared using cytobrush cytology method with PMN threshold of >8% to diagnosis SCE in the period between 28 and 41 days after birth. Furthermore, they reported that the use of endometrial thickness might be affected by several factors like the position of the uterine horn and position of the probe during ultrasonic measurements and developing a lack of the precise measurement during the examination.

2.9.5 Endometrial biopsy

Endometrial biopsy is considered the standard and optimal diagnostic ways for endometritis diagnosis (Gilbert *et al*., 2005; Sheldon *et al*., 2006). Although, the high accuracy of this technique but most of clinicians and veterinarians do not prefer this method because it needs more expensive equipment to achieve it, more times to

get results (Sheldon *et al*., 2004) and some studies reported the adverse effect of the method on predict fertility for these cows and the difficulty to perform this method in some cases (Gilbert *et al*., 2005).

2.10 Risk factors of endometritis

There are many factors may be contributed to increased the chance of endometritis occurrence in postpartum cows like calving problems, retained placenta and body condition score.

2.10.1 Dystocia and retained placenta

In cattle, dystocia is often accompanied with many post-partum disorders, such as retained placenta and impaired uterine involution, and that develop endometritis (Correa *et al*., 1993). Moreover, abnormal calving increases indirectly the chance for the development of both clinical and subclinical endometritis by increasing the probability of uterine infection like metritis with (Hossein *et al*., 2011). dystocia can induce severe trauma of the pelvic canal and also allow to the introduction of huge bacteria into the uterus and develop endometritis (Bruun et al., 2002). Also, Retained fetal membranes are considered as one of the most common predisposing factors of metritis in cows and subsequently endomrtritis in postpartum cows (Smith and Risco, 2002).

2.10.2 Body condition score

Cow's body condition score represents the quantity of subcutaneous body adipose tissue (Ferguson *et al*., 1994) and this is related to the reproductive performance. To get a good scoring, cows should be scored during the dry and early lactation interval to put a suitable plan to avoid negative energy balance during the postpartum period.

There are several factors that affect the health of postpartum beef cows and these include postpartum disorders, NEB, suckling calves and poor management. All these factors influence BCS after calving and consequently suffer from SCE, postpartum anestrus, and impairment of the reproductive performance in future. One study reported that most of the cows that have low BCS early after calving have risk factor on their reproductive performance by increasing the interval from calving to conception and pregnancy loss (Santos *et al*., 2009a). According to a recent study by Ribeiro *et al*. (2013), the optimal BCS for grazing cows was supposed to be between 3-3.25 after parturition to minimize clinical and subclinical postpartum disorders.

The NEB is considered as one of the most common reasons that have an adverse effect on the ovarian resumption after calving in dairy and suckled beef cows. NEB disrupts immune function which may further predispose cows to uterine infection

and subsequently reduce their ability to overcome postpartum uterine infection (LeBlanc 2010). Interestingly, a greater degree of negative energy balance is usually represented usually by increased non-esterified fatty acids (NEFA) and beta-hydroxybutyrate (BHBA). The level of blood NEFA factor reflects the magnitude of fat mobilization, while the level of blood BHBA reflects the oxidation of fat in the liver (LeBlanc, 2010).

CHAPTER 3

ASSESSMENT OF THREE DIFFERENT ENDOMETRIAL CYTOLOGICAL SAMPLING METHODS IN POSTPARTUM BEEF COWS

3.1 Introduction

Cows are exposed to several types of microbial contamination after parturition that can cause uterine diseases and leads to severe economic losses (Sheldon *et al*., 2006). Uterine diseases can be classified as puerperal metritis, clinical metritis, clinical endometritis and subclinical endometritis (Sheldon *et al*., 2006). Subclinical endometritis (SCE) is prevalent in high producing dairy cows and, it has been associated with decreased pregnancy per insemination, extended calving interval to pregnancy and increased culling rate (Gilbert *et al*., 2005). The precise diagnosis of cows suffering from endometrial diseases is hampered by the lack of consensus on an acceptable definition of endometritis (Gilbert *et al*., 2005; Sheldon *et al*., 2006). A huge portion of the problem is that most cows experience some degree of endometritis during normal uterine involution. SCE is an inflammation of the endometrium without the presence of mucopurulent exudates accumulating in the vagina (Sheldon *et al*., 2006). SCE is also termed as 'cytological endometritis' (Gilbert *et al*., 2005; Dubuc *et al*., 2010).

Despite the fact that transrectal palpation of the uterus is the most common means of diagnosing postpartum metritis and clinical endometritis, there is an agreement that this method lacks the accuracy to identify cows with subclinical endometritis and subsequent reduced fertility (LeBlanc *et al*., 2002; Runciman *et al*., 2008). Several methods are now used for the detection of SCE, however, only a few of these methods can be performed to collect endometrial cells. Endometrial and inflammatory cells may be collected by using a guarded cotton swab (Studer and Morrow 1978), uterine biopsy (Bourke *et al*., 1997), uterine lavage (Gilbert *et al*., 2005), cytobrush (Kasimanickam *et al*., 2004). Both cytobrush (CB) and low volume fluid (LVF) are less invasive than uterine biopsy (Kasimanickam *et al*., 2005). Application of CB is less harmful than LVF, and LVF method also extends the time required to get the samples; 17% failure to get back saline and increases the alteration of cells harvested by LVF method (Kasimanickam *et al*., 2005). However, Barlund *et al*. (2008) described CB as the most reliable method of diagnosing endometritis in cattle.

The biggest problem associated with the diagnosis of SCE is that no consensus has been reached by previous studies about the cut off values of PMN % used to differentiate diseased from healthy cows. Furthermore, the differences in the timing of sampling and the diagnostic tests used for endometritis have been variable, making comparisons among studies almost impossible. Different threshold values for the proportion of PMN have been suggested, these vary from 5 to 18%

(Kasimanickam *et al*., 2004; Gilbert *et al*., 2005; Barlund *et al*., 2008). Most of the previous studies about SCE were achieved in dairy cows and few studies were conducted in beef cows. The occurrence of SCE in beef cows in Malaysia is unknown. Thus the objective of the present study was to evaluate different endometrial cytology methods to obtain endometrial samples, to determine the occurrence of SCE in postpartum beef cows and to provide recommendations regarding the employment of these techniques.

3.2 Materials and methods

3.2.1 Animals

The study population involved all calving cows except those that were found suffering from clinical postpartum disorders. The study was conducted initialy in 48 beef cows (Brangus and Kedah-Kelantan breeds), during 3-4 weeks post calving. The study was conducted at the University Agriculture Park, UPM farm between October 2015 and July 2016. The beef farm is located in Serdang, Selangor (average temperature 28 ℃ and relative humidity about 70%) and has about 100 beef cows of different breeds. The cows are between 3 and 7 years old, with body condition scores (BCS) ranging from 2.5 to 4 (325-480 kg). The cows were managed under a free grazing system and fed with concentrated feed that comprised alfalfa, corn silage, beet pulp, cottonseed, soya bean, corn, and barley. Individual animal data such as history of calving, breed and parity were recorded. The farm used bulls with good fertility and a breeding soundness examination is performed every two months.

3.2.2 Transrectal palpation

All cows were examined via transrectal palpation between weeks 3 and 4 post-calving in order to evaluate uterine involution, symmetry and position of the uterus. Cows with abnormal vaginal discharges (mucopurulent and pus discharge) were excluded from the study. Only Forty beef cows were sampled by three different endometrial cytology methods during this study.

3.2.3 Endometrial cytological sampling

Endometrial samples were taken between days 15 and 20, and days 22 and 28 postpartum to determine the number of PMN in the endometrial samples, and to determine the occurrence of SCE between days 22 and 28 postpartum. The vulva and perineum were cleaned, rinsed with water and disinfected using povidone-iodine 5% (Betadine®, MEDA Pharma S.P.A., Milan, Italy), and dried with a clean paper towel. For each cow, samples for endometrial cytology were initially collected using a cotton swab (CS), then a cytobrush (CB) and lastly low volume flush (LVF) according to procedures of previous studies (Kasimanickam *et al*., 2005; Cocchia *et al*., 2012).

3.2.3.1 Cotton swab (CS)

A sterile cotton was attached to the tip of the AI gun and the cotton and stainless steel rod combination (AI gun 65 cm length × 4 mm diameter) were then covered with a plastic sheath (Chemise Sanitaire, IMV Technologies, France) to avoid vaginal contamination. A few drops of sterile physiological saline was placed on the cotton swab before obtaining the uterine sample, lubricated with a gel (Triad Sterile Lubricating Jelly, H&P Industries Inc., Mukwonago, WI, USA), and the device introduced into the vagina. Next, a sleeved arm was introduced into the rectum to facilitate passage of the instruments through the genital tract and os cervix. Once the device has passed through the cervix, the cotton swab was exposed and turned (360 ⁰) to get cellular materials from the adjacent endometrium (body of uterus). Collected samples were then rolled 2-3 times on a clean glass slide.

3.2.3.2 Cytobrush (CB)

In this method, a sterile cytobrush Plus GT, Medscand Medical, Germany (Fig 3.1) was modified for utilization in cows (Madoz *et al*., 2013). The handle was shortened to 2 cm and threaded to enable it to be inserted into a stainless steel rod (artificial insemination gun; 65 cm × 4 mm). The cytobrush and stainless steel rod combination were then inserted into a plastic sheath (Chemise Sanitaire, IMV Technologies, and France) to avoid vaginal contamination, The same procedure that was used in the cotton swab method for sample collection was used here. The cytobrush was then withdrawn from the genital tract, and the samples were rolled 2-3 times on to a clean glass slides.

Figure 3.1 : Cytobrush used to obtain endometrial cytological samples.

3.2.3.3 LVF method

In the low volume fluid (LVF) method, 50 mL of sterile physiological saline was infused into the uterus (Kasimanickam *et al*., 2005) using a plastic sheath supported by an AI gun as in the other procedures described previously. After the instrument had been passed through the cervix into the uterus, the AI gun was withdrawn from the female genital tract, leaving just the plastic sheath. The other end of the plastic sheath was connected to a rubber piece in order to facilitate infusion of the saline solution using a 50 mL syringe. After the infusion of 50 mL of sterile physiological saline into the uterus, the uterus was massaged for 20 seconds and then retracted to recover the fluid by negative pressure aspiration into a syringe. The recovered fluid (2-5 mL) was then transferred into a test tube and placed in an ice box until used, usually within 3 hrs. Endometrial fluid was centrifuged at 3,000 rpm for 5 minutes as reported by Kasimanickam *et al*. (2005). A drop of the sediment was streaked onto a clean glass slide and air dried.

3.2.4 Preparation and evaluation of the slides

All slides were fixed with methanol for 30 minutes and stained with 5% Giemsa stain (Appendix A) for three minutes and dried (Madoz *et al*., 2013). All the slides were evaluated by selecting ten high power fields (HPF) to determine the total cellularity of endometrial cells (epithelial cells and PMN) and cellular morphology (percentage of intact and distorted cells). All slides were also evaluated by counting 300 cells at 400 × magnification (Leitz Labourlux-S, Wetzlar, Germany) to determine the PMN %. Forty slides were selected randomly and blinded to the pathologist to get intraobserver repeatability and reduce biases. Two PMN threshold values (≥ 5% and ≥ 8%) were used to determine the occurrence of SCE in the farm between 22 and 28 days postpartum (Gilbert *et al*., 2005; Barlund *et al*., 2008; Plontzke *et al*., 2010; Madoz *et al*., 2013; Ricci *et al*., 2015).

3.2.5 Statistical analysis

All the numerical data were tested by using the Kolmogorov–Smirnov test for normal distribution using SPSS version 20; IBM Corporation, NY, USA. Since the data were not of normal distribution, it was analyzed using Kruskal-Wallis test and Mann-Whitney test. The level of statistical significance was set at $P < 0.05$. The intraobserver Reliability between two examiners was calculated by using Excel 2007. Kappa statistic was used to assess the agreement among the three cytological methods for SCE diagnosis. The value of agreement was poor if $k \leq 0.2$, fair if <0.2 $k <0.4$, moderate if $0.4 <k <0.6$, substantial if $0.6 <k <0.8$ and good if k is ≥ 0.8 (Cocchia *et al*., 2012).

3.3 Results

Out of a total of 48 cows that calved between October 2015 and July 2016, 4 cows were excluded from the study due to clinical endometritis with acute mucopurulent discharge which was examined during rectal palpation. Additionally, 2 cows were excluded due to the failure of passage through the cervix during cytological sampling at week 4 post calving, while fluid could not be recovered from 2 cows by LVF. The intraobserver repeatability between the two examiners to the slides was good (IRR= 0.82). The duration of sample collection for endometrial cytology was shorter (2-3 min.) in CB and CS than LVF (6-10 min.) method. All samples of endometrial cytology were obtained from cows, first with a cotton swab, then with the cytobrush, and finally followed by LVF (Figures 3.2, 3.3 and 3.4).

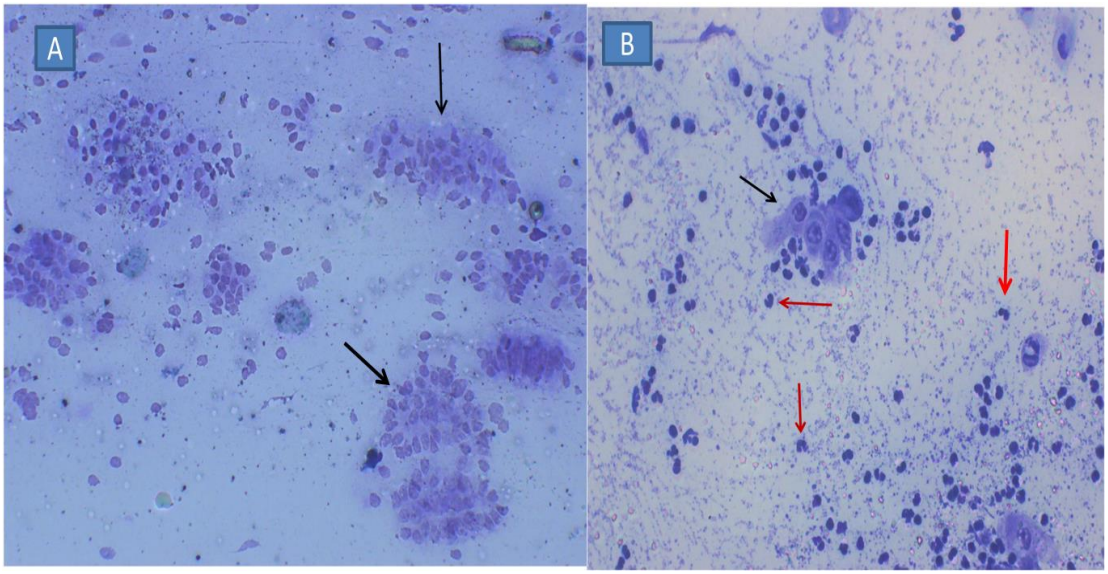

Figure 3.2 : Cytology smear obtained by CB from a healthy cow (A) and SCE cow (B), stained with Giemsa. The black arrows show endometrial cells, and red pointed neutrophil (400x).

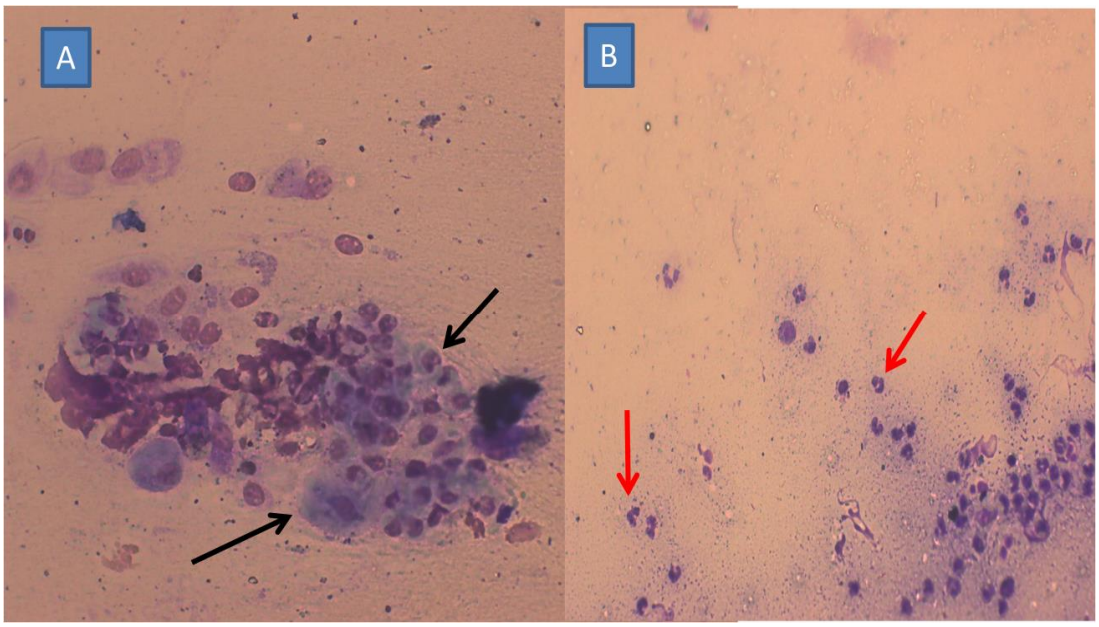

Figure 3.3 : Cytology smear obtained by CS from a healthy cow (A) from SCE cow (B), stained with Giemsa. The black arrows show endometrial cells and red pointed neutrophil (400x).

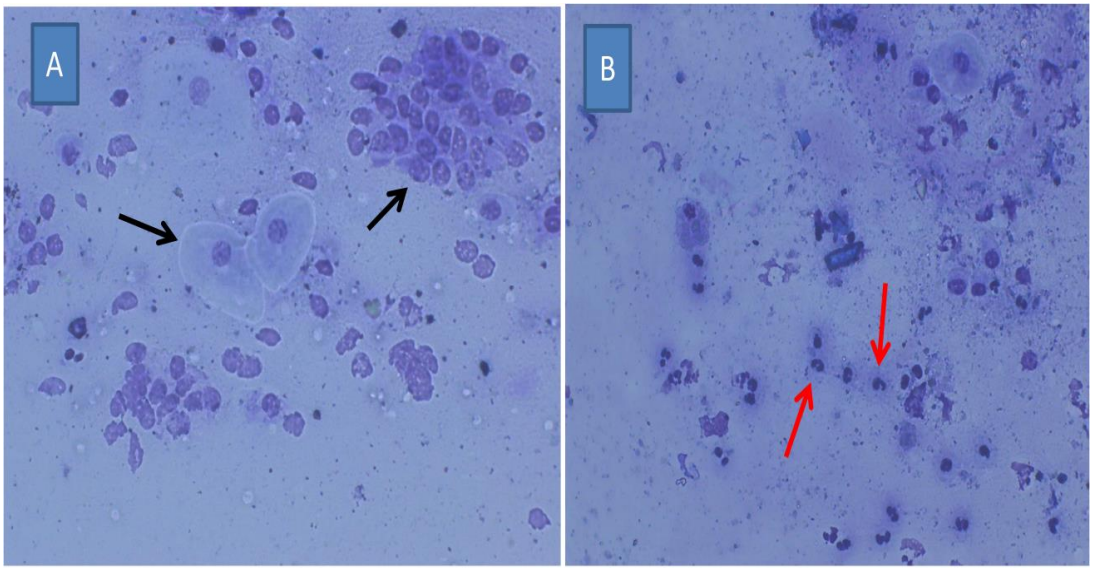

Figure 3.4 : Cytology smear obtained by LVF from a healthy cow (A) SCE cow (B) stained with Giemsa. The black arrows show endometrial cells and red pointed neutrophil (400x).

The overall average PMN by CB method at weeks 3 and 4 was higher (11.3%; $P < 0.05$) than the overall average of PMN in CS and LVF (7.0 and 6.0), respectively. The mean PMN obtained using CB technique was higher at week 3 (16.9 cells HPF^{-1}; $P < 0.05$) than week 4 (5.6 cells HPF^{-1}), while that of CS (11.02 cells HPF^{-1}) at

week 3 was higher ($P < 0.05$) than week 4 (3 cells HPF^{-1}). Similarly, the mean PMN obtained from LVF at week 3 was also higher (8.65 cells HPF^{-1}; $P < 0.05$) than week 4 (3.5 cells HPF^{-1}) (Table 3.1). At week 3, the mean PMN obtained by using CB was higher ($P < 0.05$) than CS and LVF methods and the average of PMN from CS was greater ($P < 0.05$) than LVF method. At week 4 after calving, the mean PMN obtained by CB was higher ($P < 0.05$) than the other methods; CS and LVF, while the LVF had a slightly higher value than the CS which was not significant (Table 3.1).

Table 3.1 : Mean ± SEM of PMN/HPF by three different endometrial cytological methods at week 3 and 4 postpartum.

Method	Week 3	Week 4	Mean
CB	16.92 ± 1.1 $^{a\,A}$	5.67 ± 0.3 $^{b\,A}$	11.3 ± 0.53 ^{A}
CS	11.02 ± 0.62 $^{a\,B}$	3 ± 0.28 $^{b\,B}$	7 ± 0.37 ^{B}
LVF	8.65 ± 0.71 $^{a\,C}$	3.35 ± 0.32 $^{b\,B}$	6 ± 0.35 ^{B}

CB = Cytobrush, CS= Cotton swab, LVF= Low volume fluid, PMN= Polymorphonuclear leukocytes.
^{ABC} different superscripts letters within columns indicate a significant difference at $P < 0.05$.
^{ab} different superscript letters within rows indicate a significant difference at $P < 0.05$.

The study also showed that the mean number of total endometrial cells within the smear was significantly ($P < 0.05$) highest (58.55 cells/HPF) in CB method than CS and LVF methods, while, the total cells obtained from the CS method (39.7) was higher insignificantly than LVF (36.6) (Table 3.2). The mean intact cells in the CB and CS methods were higher (62.4 % and, 61.9 %; $P < 0.05$) than the LVF method (52.4 %). The mean number of distorted cells (missing cell membrane, nuclus or cytoplasma) was highest (47.5 %; $P < 0.05$) in LVF method than the other two methods, while the CS method had insignificantly more average distorted cells (Fig. 3.5) than CB (Table 3.2).

Table 3.2 : Mean ± SEM of endometrial cells/HPF and quality (%) of endometrial cells recovered by three endometrial cytological methods.

Method	Mean ± SEM	Intact cells %	Distorted cells %
CB	58.55 ± 1.41 ^{a}	62.4 ^{a}	36.7 ^{a}
CS	39.78 ± 1.21 ^{b}	61.9 ^{a}	37.8 ^{a}
LVF	36.65 ± 1.30 ^{b}	52.4 ^{b}	47.5 ^{b}

CB = Cytobrush, CS= Cotton swab, LVF= Low volume fluid and HPF= High power field.
^{ab} different superscripts letters within columns indicate a significant difference at $P < 0.05$.
Intact cells: normal endometrial cells; distorted: abnormal endometrial cells.

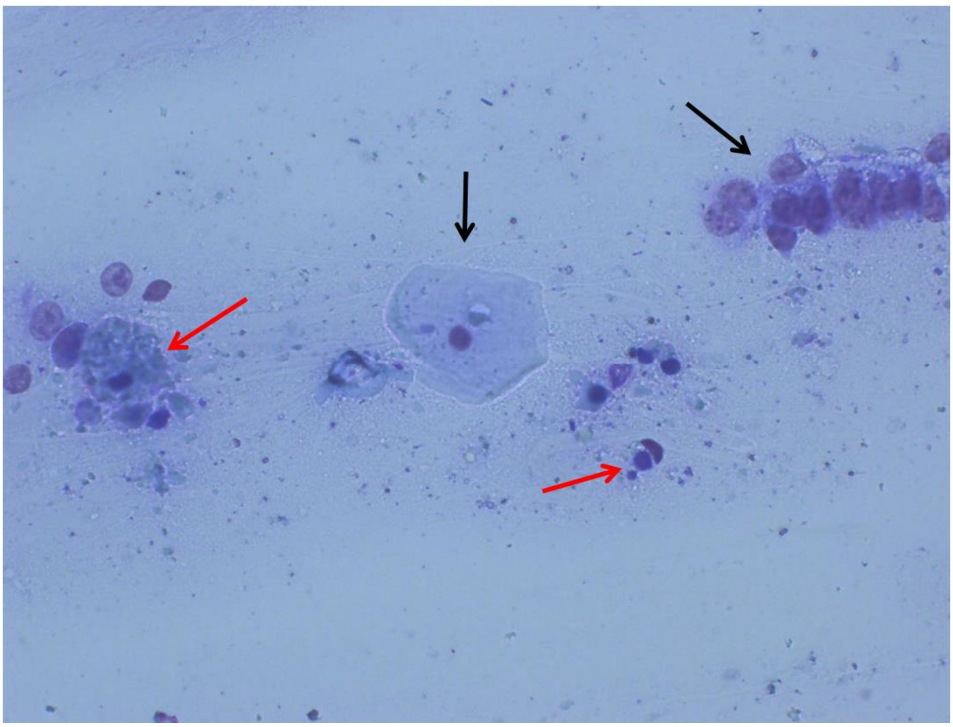

Figure 3.5 : Cytology smear obtained by CB from a healthy cow stained with Giemsa.
The black arrows show intact endometrial cells, red arrows indicate to distorted cells (400x).

The diagnosis of SCE using two thresholds of PNM% (≥ 5and ≥ 8 %) by different cytological methods are presented in (Table 3.3). The CB method had a higher occurrence of SCE at the threshold of ≥ 5 % (50%) and lower occurrence at threshold of ≥ 8 % (12.5%). The LVF method had an occurrence of 20% and 10%, while the CS method had an occurrence of 12.5 % and 7.5% at PMN threshold levels of ≥ 5 and ≥ 8, respectively. Due to the low rate of PMN cells obtained during this study, especially at week 4, decreased occurrence of SCE was noticed in these cows.

Table 3.3 : Occurrence of SCE diagnosed by using two thresholds of PMN % by different cytological methods week 4 postpartum.

Method	Occurrence of SCE	
	≥5 % PMN	≥8 % PMN
CS	12.5 %	7.5 %
CB	50 %	12.5 %
LVF	20 %	10 %

SCE=Subclinical endometritis, CB= Cytobrush, CS= Cotton swab, LVF= Low volume fluid method.

The agreement between endometrial cytological methods, sensitivity and specificity by using ≥ 5 % and ≥ 8 % PMN (22-28 d) is tabulated in Table 3.4.

The present study chose CB as a standard method to diagnosis SCE in cows because this method can pick up more endometrial and PMN cells during endometrial cytological samplings. And also many previous studies selected CB as the reference and the most reliable method of diagnosis of endometritis in cows (Kasimanickam *et al*., 2005; Barlund *et al*., 2008).

Table 3.4 : Comparison of LVF and CS techniques using ≥ 5 % and ≥ 8 %PMN (22-28 d) threshold values with CB technique as the reference diagnostic test for SCE.

Method	Threshold %	Sensitivity	Specificity	Kappa (P value)
LVF	5 %	20 %	100 %	0.21 (< 0.05)
CS	5 %	15 %	100 %	0.15 (< 0.05)
LVF	8 %	60 %	100 %	0.72 (< 0.05)
CS	8 %	40 %	100 %	0.53 (< 0.05)

CS= Cotton swab, LVF= Low volume fluid method. Kappa: agreement test among cytological methods

3.4 Discussion

Based on the results observed in this study, the duration of sample collection for endometrial cytology was shorter in CB and CS (2-3 min.) than LVF (6-10 min). Kasimanickam *et al*. (2005) and Cocchia *et al*. (2012) reported that the time needed to get uterine samples by CB was appropriate and quicker than LVF. Barlund *et al*. (2008) had selected CB as the reference endometrial cytological detection test because it is the most reliable method in the diagnosis of SCE. Besides that, during the CB procedure, only one trained person is needed to get an endometrial cytological sample, whereas, LVF method always requires two people to get a uterine specimen. In the present study eight cows were excluded because uterine samples could not be obtained from them due to various reasons stated previously. Unsuccessful attempts during sample collection were also reported with LVF technique resulting in a failure rate of 17% of all attempts to recover uterine fluid in Holstein cows (Kasimanickam *et al*., 2005). All samples of endometrial cytology were obtained from cows, first with a cotton swab, then with the cytobrush, and finally followed by LVF. This is because LVF may induce uterine irritation (Brook, 1993), thus CS and CB were done first. Furthermore, the amount of normal saline injected into the uterus in LVF method might also affect the PMN % and develop a false result from CB and CS methods later. Interestingly, there is an argument among studies about the possibility of irritation of the endometrium by the fiber nylon of the brush during CB sampling (Cocchia *et al*., 2012).

The results of this study showed a dramatic decrease in mean number PMN with the advancement of the postpartum period due to infiltration of PMN during physiological events at calving, which gradually declined over the process of the uterine involution. This finding is in agreement with previous studies that showed a reduction in the mean of PMN as the postpartum period approached the completion of histological involution (Gilbert *et al*., 1993; Kasimanickam *et al*., 2005), which usually complete at 40 days post calving (Stevenson and Britt, 1979). The results of this study also showed that the mean PMN at weeks 3 and 4 were higher from the CB method than the other two methods. Due to the fiber nature of the cytobrush tip and the rigid nylon, the vertical position of the handle tip allowed more endometrial cells to be picked up from the surface of the uterus in comparison to the other two methods. Besides that, the CB method allows for a deeper penetration of the uterine endometrium resulting in more PMN (Martin-Hirsch *et al*., 2000). Due to the soft nature of the cotton in the CS method, the number of endometrial cells and PMN were lower than in the CB method at weeks 3 and 4 (Cocchia *et al*., 2012). At the same time, the average PMN were lower in cows through LVF method than CB during 15 and 28 days postpartum, which was due to difficulties in getting infused fluid back because the uterus was not fully involuted at this time, consequently only fewer cells were obtained (Kasimanickam *et al*., 2005).

In cytological studies, PMN % obtained from endometrial cytology samples is essential due to its effect on the occurrence of subclinical endometritis in cows (Riddle *et al*., 2007). The efficiency of uterine smears is referred by the existence of uterine epithelial cells, while the lack of smears with suitable count of epithelial cells and PMN is useless as these samples smears cannot be used in the detection of disease (Martin-Hirsch *et al*., 2000). The current study showed that all endometrial cytological smears displayed intact endometrial cellularity, and the CB technique was the one that produced a higher mean of endometrial cells in comparison to the CS and LVF methods.

The present study showed increased in number of abnormal endometrial cells (irregular cells, missing nuclus, and cytoplasma) from LVF compared with CB and CS methods. This is in agreement with previous findings by Barlund *et al*. (2008) and Kasimanickam *et al*. (2005), who believed that LVF method has a harmful effect on the integrity of the cells. There are several factors that were thought to contribute to an increased mean of distorted cells by using LVF method. The extended period (3 to 6 hr) of processing the uterine samples may affect the shape and appearance of the cells and total counts in the sample. The pH of normal saline (7.0) also has a potential reason to yield more distorted cells (Vanderwall and Woods, 2003). The centrifugation process is also another reason that can increase the possibility of obtaining distorted cells, even though 3,000 rpm for 5 minutes is considered safe for the cells. Barlund *et al*. (2008) and Kasimanickam *et al*. (2005) reported variable rate of distorted cells at centrifugation speeds of 600g (2,400 rpm) for 15 min and 766g for 5 minutes, respectively, while Gilbert *et al*. (2005) used a slower speed (1000 rpm for 7 min). The mean number of distorted cells in CB and CS methods were relatively high, which can be attributed to the nature of the cotton fiber which

resulted in adherence of the endometrial cells and destruction of the cells (Bourke *et al*., 1997). On the other hand, Cocchia *et al*. (2012) reported increased mean of distorted cells by using CB method in comparison with cotton and LVF methods. This was attributed to the nature of rigid fibers of the brush which might have induced traumatic effect to the endometrial cells during sampling resulting in the development of distorted cells (Martin-Hirsch *et al*., 2000).

Until now, there is still no consensus among previous studies about the threshold value for PMN % and the precise time (range 21 to 60 days post calving) from sample collection to the diagnosis of SCE in cows. Scholars have suggested many threshold values for the proportion of PMN, ranging from 5% to 18% (Kasimanickam *et al*., 2004; Gilbert *et al*., 2005).

The agreement among the cytological methods in diagnosis SCE; LVF, CS with CB (threshold PMN ≥ 5 %) was poor (k= 0.21) and (k= 0.15) respectively. The lack of agreement among the methods used to determine prevalence of SCE, may be due to the difference in the methods that used to collect samples. For example, cytobrush has the rigid fiber that allows picking up of more endometrial cells than the other two methods (Trimbos and Arentz, 1985). Similarly, the lack of cotton to pick up and the pressure of texture cotton during sampling and rolling of the sample on the glass slide played a vital role in the reduction of endometrial cells and PMN (Bourke et al., 1997; Trimbos and Arentz, 1985). On the other hand, most of the previous studies used the LVF procedure at the late postpartum period (after 30 days post calving) (Barlund *et al*., 2008; Gilbert *et. al*, 2005; Santos *et al*., 2009b), while the current study was conducted 3 to 4 weeks after calving. The difficulty of recovering the fluid infused into the uterus during its involution, especially at week 2 post calving (Kasimanickam *et al*., 2005; Saut *et al*., 2013), and lack of infusion fluid to cover the whole uterus (big uterus after calving) might have led consequently to the decreased mean of endometrial cells and PMN % during sampling, thus resulting in a disagreement with the others methods. For these reasons, most studies considered the cytobrush method as the fastest and most reliable endometrial cytological method to collect uterine samples (Kasimanickam *et al*., 2005; Barlund *et al*., 2008).

The present study also showed the agreement among LVF, CS and CB by using CB (threshold PMN ≥ 8 %) as a reference, which were k= 0.72 and k=0.53, respectively. The agreement between the methods by using a threshold value of ≥ 8 % was substantial and better than using a threshold of ≥ 5 %, which may be due to the general reduction of mean PMN during week 4 and a decreased in the number of cows that exceeded the threshold of 8%. Cows that depended on open grazing method had less bacterial contamination and the ability to clean their uterus early in the postpartum period consequently resulting less PMN% (Madoz *et al*., 2013). Also in the present study, we adopted the criterion of Barlund *et al*. (2008), and Ricci *et al*. (2015) i.e. >8% PMN as indicative of subclinical endometritis in dairy and beef group respectively. Although their sampling period was between 28 and 41 d postpartum, we considered that using >8% PMN as an indicator of uterine

inflammation at 25 d was a more conservative approach. Barlund *et al*. (2008) indicated a good agreement k=0.74 between LVF and CB for diagnosis of SCE in dairy cattle between 28 and 41 days postpartum. The same study also showed a sensitivity (92.3 %) and specificity (93.9 %) at a threshold value of ≥ 8 % PMN and CB as a reference (Barlund *et al*., 2008), which was little higher to what was observed in the current study. In contrast to the present study, a study by Saut *et al*. (2013) showed a high occurrence of SCE; (42.3-22.2 % and 65.4- 59.3 % at 21 and 28 days postpartum through CB and LVF, respectively, by using a threshold value of ≥ 18 % PMN , giving a sensitivity of 50-100 % and specificity of 35.3-53 %. Santos *et al*. (2009b), reported in his study that SCE was uncommon in beef cattle and had minor impacts on fertility of these cows. Besides, the occurrence of SCE in dairy cows was more than in beef cows, and it ranged from 5 to 76 % using different thresholds and diagnostic methods. A study in beef cows in the USA using a threshold value of 5.5 % through LVF method showed 88 % of cows SCE before week 2 after calving, 77 % between 2 and 7 weeks postpartum and 17 % after week 7 (Santos *et al*., 2009b). In comparison to our study, the prevalence of SCE using a threshold value of ≥5% PMN was 50 % using CB, 20 % CS and 12.5 % LVF. These differences in the prevalence of SCE may be attributed to differences in geographical area, environment, and breed. Therefore, such as studies are necessary in order to demonstrate the dynamics of PMN in postpartum beef cows and their relationship with the occurrence of subclinical endometritis in these cows by using endometrial cytology methods.

3.5 Conclusion

This study reported a higher number of PMN/HPF at week 3 than 4 post calving. In addition, the CB method proved to be the best because this method can pick up more endometrial and PMN cells during endometrial cytological samplings. The mean of intact cells was higher in CB and CS than LVF. The prevalence of SCE in present study in beef cows was lower in comparison with other previous studies and the agreement between CB, CT and LVF ranged from weak to moderate. Depended on the current study the endometrial threshold value ≥ 8% was better than ≥ 5 % to determine the SCE occurrence in the farms between 20 and 30 days postpartum. The cytobrush method was the most reliable endometrial cytological method to collect uterine samples compared it with other two methods.

CHAPTER 4

AGREEMENT AMONG BACTERIOLOGICAL FINDINGS, VAGINAL DISCHARGES, AND ENDOMETRIAL CYTOLOGY FOR ENDOMETRITIS DETECTION IN POSTPARTUM BEEF COWS

4.1 Introduction

Most postpartum cows suffer from pathogenic microorganism contamination of the uterus 2 to 3 weeks after parturition, and more than 80% of the cows after calving are exposed to uterine contamination by *Escherichia coli*, *Fusobacterium* sp., *Arcanobacterium pyogenes*, *Streptococcus* sp., *Staphylococcus* sp., *Clostridium* sp., *Pasteurella multocida*, and *Bacteroides* sp. (Sheldon *et al.*, 2006). McDougall *et al.* (2011) isolated and registered more than 35 bacteria species that increased the risk for genital tract infection after parturition in cows.

Uterine infection can be categorized into puerperal metritis, clinical metritis, clinical endometritis, and subclinical endometritis (Sheldon *et al.*, 2006). Clinical endometritis (CE) is known as endometrial inflammation with purulent or mucopurulent discharge; this disease can be detected 21 days after calving and is not associated with clinical signs of sickness (Sheldon *et al.*, 2006). The correct diagnosis of CE depends on the presence of vaginal discharge (LeBlanc *et al.*, 2002). Clinical endometritis can be diagnosed using a vaginoscope, meter check device, vaginoscope, or gloved hand (Leutert *et al.*, 2012). The term "purulent vaginal discharge" has been adopted in place of the term CE because the presence of abnormal genital discharge does not necessarily indicate endometrial inflammation (Dubuc *et al.*, 2010). Subclinical endometritis (SCE) is known as the endometrial inflammation of the uterus without mucopurulent material accumulation in the vagina (Sheldon *et al.*, 2006). SCE has also been identified as cytological endometritis (Gilbert *et al.*, 2005). Dubuc *et al.* (2010) described cytological endometritis as an elevated ratio of polymorphonuclear neutrophils (PMN) in endometrial cytology samples obtained by cytobrush or low-volume uterine lavage. Assessment of cows suffering from endometrial infections is hampered by the lack of consensus on an acceptable definition of endometritis in cows (Gilbert *et al.*, 2005; Sheldon *et al.*, 2006) and simple, effective diagnostic methods. Despite that transrectal palpation of the uterus is commonly used to diagnose uterine diseases, this technique cannot accurately identify cows with endometritis that lead to reduced fertility (Runciman *et al.*, 2008).

SCE can be detected using several methods, some of which can be performed during collection of endometrial cells. In these techniques, endometrial and inflammatory cells may be collected by using a guarded cotton swab (Studer and Morrow, 1978), uterine biopsy (Bourke, *et al.*, 1997), uterine lavage (Gilbert *et al.*, 2005), cytobrush (Kasimanickam *et al.*, 2004). However, the significant challenge in SCE diagnosis is

the absence of consensus among studies regarding the sampling time and cutoff values to differentiate diseased from healthy cows. Scholars have suggested many threshold values for the proportion of PMN, ranging from 5% to 18% (Kasimanickam *et al*., 2004; Gilbert *et al*., 2005).

The occurrence of uterine diseases in beef cows in Malaysia remains unclear, bacterial spectra, sensitivity to antibiotics, and the relationship between bacterial contamination has been rarely investigated. The present study aims to assess the relationship among bacterial contamination, vaginal discharges, and endometrial cytology findings in postpartum beef cows and to determine the diagnostic value of these parameters for detection of CE and SCE. Also to determine the sensitivity of the bacteria isolated to antibiotics.

4.2 Materials and methods

4.2.1 Animals

One hundred cows (55 Brangus and 45 Kedah-Kelantan breeds) at 20 days to 30 days post-calving period were obtained from three different private beef farms. All samples were collected from animals while cows remained at their farm of origin between June 2015 to October 2016. The beef farms are located in Serdang, Selangor where the average temperature is 28 ℃, and relative humidity is about 70%. The cows were aged 3–7 years, weighed 300–450 kg, and managed under free grazing. The cows were also fed according to field management after providing concentrated feed, which consisted of alfalfa, corn silage, beet pulp, cottonseed, soybean, corn, and barley. Individual animal data on calving history, lactation, breed, parity were recorded. The farms used many bulls with a high fertility and passed a breeding soundness examination conducted every two month. These farms used 20:1 as cow-to-bull ratio for natural mating after the postpartum period. The body condition score (BCS) of the cows was evaluated by using a 5-point scale (Ferguson *et al*., 1994). Pregnancy diagnosis for cows was achieved by using B-mode ultrasound attached with a linear probe of 5MHz frequency (Sonosite VET 180 Plus, Bothell, WA, USA) at 150 and 200 days after calving.

4.2.2 Animals physical examination

All practical examinations and scoring for clinical findings were done by the same veterinarian (author) for postpartum cows of the current study.

All cows were checked by transrectal palpation 20 to 30 days after calving to evaluate degree of uterine involution, symmetry of uterine horns, and position of the uterus relative to the pelvic brim Genital tract discharge of all cows was checked through examination of vaginal secretions by using hands covered with clean

disposable long gloves at 20 days to 30 days post-calving to identify CE cases. A four-grade system (0 = clear mucus, 1 = mucus containing flecks of pus, 2 = discharge including < 50% pus, and 3 = involving > 50% pus) was used to categorize vaginal secretions (Williams *et al*., 2005). The vulva and perineum region were washed, cleaned, sterilized with iodine, and dried using clean, sterile paper towels. The area was then lubricated (Triad Sterile Lubricating Jelly, H&P Industries Inc., Mukwonago, WI, USA). The hand covered with a long sterile disposable glove was inserted into the vagina far enough to allow observation of the nature of the fluid; if necessary, a light source was used to obtain evidence of abnormal cervical secretion (Barlund *et al*., 2008).

4.2.3 Endometrial cytological examination

Endometrial cytological samples were collected from cows by using CB as described in chapter 3 (3.2.3.2) The sample was rolled on the sterile microscopic slide (75mm×25mm) and stored in a transport medium (LABCHEM SDN.BHD, Malaysia) for bacteriological analysis. All slides were fixed with methanol for 30 min, transported to the laboratory within 3 h, stained with 5% Giemsa stain for 3 min, and dried. All of the slides were evaluated by counting 300 cells at 400× magnification (Leitz Labourlux-S, Wetzlar, Germany) to determine the percentage of neutrophils (PMN %). Endometrial threshold value ≥ 8% was used (Madoz *et al*., 2013; Ricci *et al*., 2015)) to determine the SCE occurrence in the farms between 20 and 30 days postpartum.

4.2.4 Bacterial isolation and Antimicrobial Susceptibility Testing (AST)

Bacteriological samples were transferred into sterile tubes containing thioglycolate broth as transport medium (Sterile transport media, LABCHEM SDN. BHD, Malaysia). The samples were transported to the laboratory in an ice box and immediately processed for bacteriological examination. Samples were cultured aerobically on sheep blood agar, MacConkey agar, and nutrient agar. Bacterial growth on the culture plates was scored semi-quantitatively after 18 hours of incubation at 37 ℃ for aerobic growth. Bacteria were identified based on the shape of the bacterial colony, Gram stain, hemolysis, morphology, and biochemical tests such as coagulase, oxidase, catalase, indole production, and methyl red.

All the bacterial colonies that were isolated (32 isolation) from the endometritis cows (18 cows) were suspended in the nutrient broth and incubated for 18 hrs. A concentration of 0.5 (1.5 x $\mathbf{10}^{8}$) McFarland Standards was used for bacterial suspension. A sterile cotton swab was soaked in the incubated bacterial suspension and then was rolled on the whole surface of Muller-Hinton agar medium and was equally covered with suspension bacteria. Six antimicrobial discs (Oxoid™, Thermo Fisher scientific, USA): Enrofloxacin 5 µg, Tetracycline 15 µg, Streptomycin 10 µg, Penicillin G 10 iu, Amoxicillin 10 µg, and Neomycin 75 µg were used to do sensitivity test by putting these discs on the surface of the Muller-

Hinton agar plates (Appendix B). The zones of growth inhibition around each of the antibiotic discs are measured to the nearest millimeter. The diameter of the zone is related to the susceptibility of the isolate and to the diffusion rate of the drug through the agar medium (Jorgensen and Ferraro, 2009).

4.2.5 Statistical analysis

Clinical findings and all data were recorded and encoded into excel software (Excel 2007, Microsoft Office Corporation, Redmond, WA, USA). The data included BCS, parity, date of parturition, type of calving, postpartum diseases, vaginal clinical findings (discharge score), cytological examination findings, bacteriological isolation results, and reproductive performance of cows. All the statistical methods were performed by SPSS software (version 18.0, IBM SPSS Inc., Chicago: USA) and Excel 2007. The occurrence of SCE and CE was determined based on clinical examination results, vaginal discharges, and endometrial cytological samples. The agreement among vaginal discharge grades, the percentage of PMN, and bacteriological findings were examined using Kappa analyses (Santos *et al*., 2009b). Chi-square analysis was used to compare the bacteriological results of healthy and infected cows. Moreover, the relative risk factor for the occurrence of SCE and CE was measured, considering isolated bacteria (*E. coli*, *Staphylococcus*, and *Bacillus*) by using SPSS software consequently; just isolated bacteria with P value < 0.10 were considered to have significant effects and included in regression model. A binary logistic regression model was used to analyze risk factors for the occurrence of SCE and CE. These factors included infection with *E. coli* (0 = no infection, 1 = infection), infection with *Bacillus* spp. (0 = no infection, 1 = infection), infection with *Staphylococcus* spp. (0 = no infection, 1 = infection), calving (0 = normal, 1 = dystocia), and BCS (0 = ≥ 3, 1 = < 3), parity (0= primiparous, 1= multiparous).

Reproductive performance parameters were reported by calculating the percentage of pregnancy rate at 150 and 200 days postpartum diagnosed by using ultrasound technique. A Cox regression model for non-pregnancy within 200 days post-calving was established using the following grading system: CE (0 = CE, 1 = no CE), SCE (0 = SCE, 1 = no SCE), infection with *E. coli* (0 = no infection, 1 = infection), infection with *Bacillus* spp. (0 = no infection, 1 = infection), infection with *Staphylococcus* spp. (0 = no infection, 1 = infection), calving (0 = normal, 1 = dystocia), and BCS (0 => 3 , 1 =< 3) (Madoz *et al*., 2013). The level of significance was set at $P<0.05$ for all statistical analyses, and the CI was set at 95%.

4.3 Results

One hundred cows enrolled in this study, 18 cows were excluded because of the presence of clinical mastitis (5 cows), sold (7 cows), lameness (3 cows) and inadequate endometrial cytological sampling (3 cows). The remaining 82 cows were identified as Brangus (54.87%, 45/82), Kedah–Kelantan (45.12%, 37/82), primiparous (29.2%, 24/82), and multiparous (70.8%, 58/82). Calving assistance

(dystocia) 11% (9/82), retained placenta 4.8% (4/82), and twins 2.4% (2/82) were recorded in cows of the current study.

4.3.1 Vaginal examination

Vaginal examination on postpartum days of 20-30 showed nine cows (11%) with abnormal vaginal secretions. Four cows had a vaginal secretion score of 1, four cows were scored 2, and one cow was scored 3.

4.3.2 Bacterial isolation

Regarding bacterial isolates, 20 different microorganisms were identified among the total 114 bacterial isolates obtained from 56 of the 82 (68%) postpartum cows; no bacterial isolates were obtained from the remaining 26 cows. Multiple bacterial species were isolated from 38 of the 56 cows (68%), whereas a single bacterial species was isolated from the remaining 18 cows. Bacterial isolates are shown in Table 4.1. *E. coli*, *Bacillus* sp., *Staphylococcus* sp., and *Streptococcus* sp. accounted for 21.9 % (25/114), 12.28% (14/114), 6.14% (7/114), and 13.15 % (15/114) of the bacteria species isolated, respectively (Table 4.1). *Escherichia coli* was the most commonly isolated bacteria from SCE (42.85%; 6/14), and CE cows (38.88%; 7/18), which was significantly higher ($P < 0.05$) than healthy cows (14.6%; 12/82). The commonly isolated bacteria from CE cows were *E. coli* (38.88%), *Staphylococcus aureus* (22.2 %), *Bacillus* sp. (11.11%), *Streptococcus bovis* (5.5%), *Proteus* sp. (5.5.1%), and *Arcanobacterium pyogenes* (5.5. %). *Staphylococcus aureus* percentage was higher ($P < 0.05$) in CE (22.2 %) than in healthy cows (1.21 %). The most common bacterial species present in SCE cows were *E. coli* (42.85%) and *Bacillus* sp. (14.2 %). Moreover, no bacteria were isolated from two cows (2/9) with SCE.

Table 4.1 : List of bacteria isolated from healthy, CE, and SCE cows.

Species of bacteria isolated	Isolation rate		
	Healthy Cows	CE* Cows	SCE** Cows
Escherichia coli	12 (14.63 %)[a]	7 (38.88%)[b]	6 (42.85 %) [b]
Bacillus spp.	10 (12.19 %)	2 (11.11 %)	2 (14.28 %)
Staphylococcus schleiferi	2 (2.43 %)	-	1 (7.14 %)
Staphylococcus aureus	1 (1.21 %) [c]	4 (22.22 %) [d]	-
Streptococcus bovis	3 (3.65 %)	1 (5.5 %)	1(7.14 %)
Streptococcus pneumonia	4 (4.87 %)	-	1(7.14 %)
Streptococcus agalactia	2 (2.43 %)	-	-
Streptococcus zooepidemicus	3 (3.65 %)		-
A. pyogenes	1 (1.21 %)	1 (5.5 %)	-
Proteus spp.	1 (5.5 %)	1 (5.5 %)	-
Enterobact *faecium*	4 (4.87 %)	1 (5.5 %)	-
Alcaligenes faecalis	5 (6.09 %)	-	-
Enterobacter cloacae	5 (6.09 %)	-	1(7.14 %)
Klebsiella pneumoniae	3 (3.65 %)	1 (5.5 %)	1(7.14 %)
Acinetobacter baumannii	4 (4.87 %)	-	1(7.14 %)
Acinetobacter lwoffii	4 (4.87 %)	-	-
Acinetobacte rcalcoaceticus	5 (6.09 %)	-	-
Chromobacterium	4 (4.87 %)	-	-
Pantoea agglomerans	4 (4.87 %)	-	-
Lactobacillus fermentus	3 (3.65%)	-	-
Pasteurella spp.	2 (2.43 %)	-	-
Total bacterial isolation	82 (100 %)	18 (100 %)	14 (100 %)

*CE: Clinical endometritis, **SCE: Subclinical endometritis.

[a b c d]Different lowercase letters in the same row indicate significant differences ($P < 0.05$).

4.3.3 Endometrial cytology

All cows were sampled by cytobrush, of which, nine cows manifested SCE (12.3%, determined by using ≥ 8% PMN as threshold value and vaginal discharge score = 0). The average PMN % on the slides ranged from 0 to 29%. The agreement among endometrial examination, PMN %, and vaginal discharge score (0–3) was moderate ($k = 0.48$, $P < 0.05$; Table 4.2). There is a poor agreement between vaginal discharge score (0–3) and bacteriological finding ($k = 0.032$, $P > 0.05$) and between bacterial finding and PMN ($k = 0.15$, $P < 0.05$) (Table 4.2).

Table 4.2 : Agreement among endometrial cytology, bacterial isolation and vaginal discharges for postpartum cows.

		Bacterial Isolation		Kappa
		Positive	**Negative**	
Cytobrush	≥ 8 % PMN	16	2	K=0.15
	< 8 %PMN	40	24	P<0.05
		Vaginal Discharges		
		Positive	**Negative**	
Bacterial isolation	Positive	7	49	K=0.032
	Negative	2	24	P>0.05
		Vaginal Discharges		
		Positive	**Negative**	
Cytobrush	≥ 8 % PMN	7	9	K=0.48
	< 8 %PMN	2	64	P<0.05

Beef cows with <8% and ≥ 8% of polymorphonuclear leukocytes (PMN) in endometrial cytology samples, negative (score 0) or positive (scores 1, 2, and 3) gross vaginal inflammation score, and the presence or absence of bacterial contamination at days 20–30 post calving. The Kappa statistic (K) is a measure of the level of agreement between the tests, where 1 = complete agreement and 0 = no agreement.

4.3.4 Risk factors affecting the occurrence of CE and SCE

Table 4.3 shows the significant relative risk factor of *E. coli* and *Staphylococcus aureus* for the occurrence of SCE and CE. *E. coli* was the major bacteriological risk factor for the occurrence of SCE (odds ratio (OR) = 8.6; 95% CI = 1.89–39.68; P < 0.05) and CE (OR = 7; 95% CI = 1.05–47.1; P < 0.05) (Table 4.4). *E. coli* and *Staphylococcus aureus* were the major bacteriological risk factor for the occurrence of CE (OR = 10.16; 95% CI = 1.66–61.91; P < 0.05). Calving assistance was considered a risk factor for CE occurrence in beef cows (OR = 10.6; 95% CI = 1.48–76.09; P < 0.05), whereas BSC was a risk factor only for SCE occurrence (OR = 11.7; 95% CI = 1.24–113.32; P < 0.05). Other isolated bacterial species and parity did not have obvious risk effects on the occurrence of CE and SCE (Tables 4.3 and 4.4).

Table 4.3 : Bacteriologic relative risk factors for cows diagnoses with clinical endometritis and subclinical endometritis between 20 and 30 days postpartum.

Factor	Relative Risk	95 % Cl	P Value
Diagnosis CE*			
Bacillus	1.3	0.314-5.73	P > 0.05
E coli	9.9	1.33-26.66	P < 0.05
Streptococcus sp.	2.1	0.35-13.08	P > 0.05
*Staph*ylococcus aureus	8.9	3.2-24.6	P < 0.05
Trueperella pyogenes	4.4	0.959-20.53	P > 0.05
Diagnosis SCE**			
E coli	6.1	1.70-21.97	P < 0.05
Bacillus	1.4	0.34-6.15	P > 0.05
Streptococcus sp.	1.2	0.28- 5.08	P > 0.05

*CE: Clinical endometritis, ** SCE: Subclinical endometritis.

Table 4.4 : Results of binary logistic regression analysis for the risk of SCE and CE in cows examined at 20 days to 30 days postpartum.

Variables*	Odds Ratio	95 % Cl	P Value
Diagnosis CE			
E coli	7.0	1.05-47.2	P < 0.05
Staphylococcus aureus	10.16	1.66-61.91	P < 0.05
Parity	0.69	0.154-3.08	P > 0.05
Calving assistance	10.6	1.48-76.09	P < 0.05
BCS	2.5	0.32-19.4	P > 0.05
Diagnosis SCE			
E coli	8.6	1.89-39.68	P < 0.05
BCS	11.7	1.24-113.32	P < 0.05
Calving assistance	3.0	0.53-16.6	P > 0.05
Parity	1.93	0.56-6.58	P > 0.05

Variables*: CE: clinical endometritis, SCE: Subclinical endometritis, OR:0dds ratio, BCS: body condition score, infection with E coli (0= infection:1= no infection), infection with bacillus (0= infection:1= no infection), infection with Staphylococcus (0= infection:1= no infection), calving assistance(0=assisted calving: 1=normal calving): BCS :(0= < 3: 1= ≥3), parity : (0=primparous: 1=multiparous).

4.3.5 Reproductive performance

Cox regression model analysis showed that SCE, CE, and bacterial agents did not significantly affect the risk for non-pregnancy at 200 days after calving (P > 0.05) (Table 4.5). The pregnancy rates at 150 and 200 days postpartum were 35.3%

(29/82) and 45.12% (37/82), respectively. The percentages of pregnant cows at 150 days postpartum were 37.5% (24/64), 33.3% (3/9), and 22.2% (2/9) in healthy, SCE, and CE groups, respectively. The percentages of pregnant cows at 200 days postpartum were 46.8% (30/64), 44.4% (4/9), and 33.3% (3/9) in healthy, SCE, and CE groups, respectively (Table 4.6).

Table 4.5 : Results of Cox regression for the hazard of non-pregnancy within 200 days in cows examined 20 days to 30 days after calving.

Factors*	**Non- pregnancy**		
	HR	**CL 95%**	**P value**
CE	1.49	0.66-3.34	P > 0.05
SCE	1.24	0.5-2.94	P > 0.05
E coli	1.3	0.63-2.75	P > 0.05
Bacillus	0.51	.63-2.75	P > 0.05
Staphylococcus	0.9	0.28-2.91	P > 0.05
Calving assistance	.51	0.26-1.22	P > 0.05
BCS	0.76	0.42-1.37	P > 0.05

*Factors: CE: clinincal CE: clinincal endometritis, SCE, Subclinical endometritis, HR, hazard ratio, BCS: body condition score, CE(0= CE: 1= no CE diagnosed), SCE(0= SCE: 1= no SCE diagnosed), infection with E coli (0= infection:1= no infection), infection with bacillus (0= infection:1= no infection), infection with Staphylococcus (0= infection:1= no infection), calving assistance(0=assisted calving: 1=normal calving), BCS: (0= < 3: 1= ≥3).

Table 4.6 : Reproductive performance of beef cows.

Variable		Total (%)	healthy	SCE*	CE**
Number of cows		82	64	9	9
Pregnancy rate	At 150 days post calving	29/82 (35.3)	24/64 (37.5)	3/9 (33.3)	2/9 (22.2)
	At 200 days post calving	37/82 (45.1)	30/64 (46.8)	4/9 (44.4)	3/9 (33.3)

* SCE: Subclinical endometritis, **CE: Clinical endometritis.

4.3.6 Antimicrobial Susceptibility Testing (AST)

Different species of bacteria isolated from the uterus of postpartum cows were exposed to the *in-vitro* antibiotic sensitivity test using six different antimicrobials, and are presented in Table 4.7. The results showed the increased sensitivity of most isolated bacteria to enrofloxacin and tetracycline than any another antibiotic used. Most of the isolated *E coli* bacteria were resistance to Amoxicillin and Neomycin while all isolated Bacillus sp were resistance to Amoxicillin. Most of the pathogenic isolated bacteria was resistance to Neomycin.

Table 4.7 : Results of antibiotic sensitivity test for uterine swabs in CE and SCE cows.

Bacteria type	Total isolates	No. of isolates sensitive to Enfl	Tetra.	Stre.	Pen. G	Amo.	Neo.
E.coli	13	6	4	2	2	-	-
Bacillus sp	4	3	-	1	1	-	1
Staph sp	5	1	1	-	2	1	1
Strpt.sp	3	1	1	1	1	-	-
Klebsiella	2	1	1	-	-	1	1
A. pyogen	1	1	1	-	-	-	-
Proteus	1	1	-	-	-	-	1
Enterobact faecium	1	1	-	-	-	1	1
Acinetobacter	1	-	-	-	-	1	-
Enterobacter	1	1	-	-	-	1	-
Total	32	15	9	7	6	6	5
Sensitivity (%)	-	47%	28%	21.9%	18.7%	18.7%	15.6%

Enfl.- Enrofloxacin, Tetr- Tetracycline, Stre.- streptomycin, Pen. G- penicillin G, Amo- Amoxicillin, Neo.- Neomycin.

4.4 Discussion

Beef cows are the primary source of production animals for the meat industry and many problems like dystocia and postpartum problems are affecting the productivity of these cows (Diskin and Kenny, 2016). The effects of these factors on CE and SCE rates and reproductive performance were also investigated. The overall occurrence of CE was 10.97%, which is lower than the range reported by previous studies in cow farms in other countries. Most studies recorded a CE prevalence between 20% to 40% in postpartum dairy cows (Heuwieser *et al*., 2000; McDougall *et al*., 2011). The discrepancy in the results may be due to decreased post-calving problems, such as dystocia, retained placenta, and metabolic disorders. Furthermore, most cows in this study depended on grazing and had low milk production, thereby developing few stress factors and minimal exposure to uterine infection after calving. The SCE occurrence in the present study was 12.3%, which is lower than that in a previous study in beef cows (Santos *et al*., 2009b) and other studies in dairy cows (Cheong *et al*., 2011; Madoz *et al*., 2013). In one of the previous studies in beef cows, 17% of Angus cows (2-78 d postpartum) were positive to endometritis by using low-volume uterine lavage method (Santos *et al*., 2009b).

Thus far, no consensus has been established with regard to the effect of threshold value and time of uterine sampling on SCE diagnosis. SCE can be diagnosed using different cut-off values, such as PMN % range of 5%–18, and various techniques using cytobrush and low-volume lavage (Barlund *et al*., 2008). Other studies depended on the thresholds of PMN % according to the effects on the reproductive performance (Cheong *et al*., 2011; McDougall *et al*., 2011). Kasmanickam *et al*. (2004) depended on >18% PMNs as threshold value between 20 -33 days postpartum and >10% PMNs between 34 and 47 days postpartum using the

cytobrush to diagnosis endometritis while Gilbert *et al*. (2005) used >5% PMNs as a significant cut-off point for diagnosis endometritis in cows using lavage between 40 and 60 days postpartum. The low SCE prevalence may be attributed to differences in geographic area, environment, and endometrial cells counted among the studies. A total of 300 cells were counted per slide in the present study, whereas 100 cells were counted in previous studies (Barlund *et al*., 2008).

E. coli was the common bacteria isolated from healthy (14.6%) and endometritic cows (38.8% in CE cows and 42.8% in SCE cows) in the period between 20 days to 30 days postpartum. These results are either similar (Santos *et al*., 2011; Sens and Heuwieser, 2013) or higher than those reported in previous studies (McDougall *et al*., 2011; Prunner *et al*., 2014a). Most studies confirmed that *E. coli* was isolated in the early period of 0 day to 15 days after calving, and the percentage gradually decreased with the advancement of the postpartum period (Werner *et al*., 2012). The present results agree with previous study demonstrating that *E. coli* and *A. pyogenes* were the common uterine pathogens in the postpartum period (Sens and Heuwieser, 2013). Werner *et al*. (2012) reported the lack of association between the abnormal vaginal discharge and *E. coli* infection in endometritic cows. Although *E. coli* is a common bacteria in the environment, specific strains of this species have been isolated from cows with uterine diseases (Sheldon *et al*., 2006). Endometrial pathogenic *E. coli* is more adherent and invasive in the endometrium compared with *E. coli* isolated from the uterus of clinically unaffected cows. These strains pathogenic *E. coli* develop diseases of endometrial surfaces such as postpartum metritis or endometritis in the bovine genital tract (Sheldon *et al*., 2006). In contrast to most studies, the present study showed a decreased number of *A. pyogenes* (one isolate) in CE cases, leading to few cases of purulent vaginal discharge (scores 2 and 3) (Zobel, 2013). However, Sens and Heuwieser (2013) reported in the previous study about bacterial isolation during postpartum period that *E coli and A. pyogenes* were the dominant bacteria that isolated from uterus between 7-24 days postpartum.

Moreover, *Streptococcus* isolation from cows in the present study was less frequent and did not severely affect CE and SCE; similarly, previous studies reported *Streptococcus* as an opportunistic bacteria in the postpartum uterus (Williams *et al*., 2005). In contrast to the present findings, Werner *et al*. (2012) reported that infection with *Streptococcus* during the early postpartum period increased the risk for the occurrence of abnormal vaginal discharge and elevated uterine PMN %. In the present study, *S. aureus* was isolated from CE cows and affected the risk factor for its occurrence. These results are consistent with previous studies, thereby confirming the serious effect of *Staphylococcus* sp. on uterine infection (Prunner *et al*., 2014b). However, the results were in contrast to other studies reporting the absence of significant effect of *Staphylococcus* on CE cows (Williams *et al*., 2005).

Most pathogenic bacteria isolated from postpartum cows with uterine diseases are *Escherichia coli, Prevotella* spp. (Sheldon *et al.,* 2006). Also, many studies isolated *Streptococcus* sp. *Staphylococcus* sp., or non-coliform aerobic gram-negative rods

(Kaczmarowski *et al.*, 2004; Jadon *et al.*, 2005). To choose a suitable and effective antimicrobial drug to treat postpartum uterine diseases, it is very important to know the sensitivity of the pathogen bacteria to antibiotics Antibiotic resistance has emerged as an essential serious problem in veterinary science and human (Takamtha *et al.*, 2013).

All of the isolated bacteria (32 isolation) from cows that had clinical and subclinical endometritis (18 cows) in the current study were tested by antibiotic sensitivity test to choose suitable and effective antimicrobial against pathogenic bacteria in these cows.

The results of antimicrobial sensitivity test in this study showed the inhibition effects of enrofloxacin on the in vitro bacterial growth, most of the isolated bacteria were sensitive to enrofloxacin and tetracycline. This result agrees with finding for another study that concluded in the inhibition role of norfloxacin for *E coli* (100 %) and *A. Pyogenes* (75.7 %) (Malinowski *et al*., 2011), However, others studies found the tetracycline the most suitable antibiotic to treat the cases of metritis in cows (Muneer *et al*., 1991; Bhat and Bhattacharyya, 2012). This result also agrees with our finding because tetracycline was the second effective antibiotic that inhibits bacterial growth in our experiment. Udhayavel *et al*. (2013) reported that a large number of isolated uterine discharges in endometritis cows were sensitive to the ceftriaxone, followed by other antibiotics such as gentamicin, enrofloxacin, and Chloramphenicol was the ineffective antibiotic to bacterial growth among them. Silva and Lobato (1998) found during their study, Arc. pyogenes was the most bacteria that sensitive to ampicillin, enrofloxacin in vitro antibiotic sensitivity test. The same previous study also reported that E coli strains were are more sensitive for chloramphenicol, enrofloxacin, gentamycin. In contrast to our results, one study concluded the failure of using enrofloxacin (1 g) to treat the cows with endometritis and combine the enrofloxacin with EDTA-Tris (buffer solution) enhance the getting positive results (Farca *et al*., 1997).

Early detection of uterine diseases and main bacterial causes of these diseases are the most common methods to prevent and control of postpartum disorders. In vivo antibiotic sensitivity test is considered a common method to choose the best treatment for uterine diseases.

This study showed the effect of calving assistance (dystocia) as a risk factor on CE (Table 4.4); the result is in agreement with previous study reporting the effect of calving assistance on the rate of possible uterine contamination after calving (Madoz *et al*., 2013). In cattle, dystocia is often accompanied with many post-partum disorders, such as retained placenta and impaired uterine involution, and that develop endometritis (Correa *et al*., 1993). Moreover, abnormal calving increases indirectly the chance for the development of both clinical and subclinical endometritis by increasing the probability of uterine infection like metritis (Ghavi *et al*., 2011). dystocia can induce severe trauma of the pelvic canal and also allow to the

introduction of huge bacteria into the uterus and develop endometritis (Bruun et al., 2002). Rogers *et al*. (2004) mentioned in one study that cows which had dystocia has more chances to suffer from uterine infections and increased culling rate in the future than those that calved without assistance. However, this result is in contrast with another study that did not find any effect of calving assistance on CE and SCE occurrence (Prunner *et al*., 2014a).

In the present study, no association was found between BCS and CE occurrence; similarly, previous studies that failed to demonstrate any effect of BCS on CE *(Potter et al.*, 2010). However, Cheong *et al*. (2011) reported that BSC significantly affected the rate of CE in cows. The present study also demonstrated the effect of BCS on SCE occurrence, which may be attributed to the effect of negative energy balance (NEB) after calving on the immunity of cows, thereby increasing the probability of uterine infection (Gilbert *et al*., 2005). Hammon *et al*. (2006) mentioned that uterine infection was accompanied with NEB, which begins before birth and continues through the early lactation; this study also reported that cows with acute NEB exhibit reduced neutrophil function and developed SCE. body energy reserves at calving consider an essential factor influencing reproductive performance in beef cattle, and it is a most important factor determining when beef heifers and cows will resume cycling after calving and response to postpartum nutrient intake (Wettemann *et al*., 2003).

In the present study, poor to moderate agreement was found among PMN %, bacteriological findings, and vaginal discharges. According to previous studies, results indicated that abnormal discharges do not necessarily indicate uterine infection (Dubuc *et al*., 2010; Westermann *et al*., 2010). The present study also showed poor agreement between bacterial findings and PMN, similar to the findings of other studies (Barański *et al.*, 2012). Not all of the bacterial species cause inflammation and infusion of PMN to the uterine endothelium because numerous bacteria may be normal inhabitants of the uterus; uterine infection after calving can also be due to other causes, such as yeast and viruses.

Reproductive performance is one of the most common economically important traits in beef production (Bormann *et al*., 2006).The current study showed minimal effects of CE, SCE, and isolated bacteria on the reproductive performance of cows because the ability of these cows to self-cured; this finding is similar to those reported in a previous study (Prunner *et al*., 2014b). However, most studies showed increased days open and days to conception in cows suffering from uterine diseases, such as CE and SCE (Elkjær *et al*., 2013; Madoz *et al*., 2013).

The decreased number of acute infection cases (scores 3) of the endometrium caused by *A. pyogenes* could result in the reduction of the acute destruction of the endometrium layer and impairment of future reproductive performance of these cows. Most of the beef cow herds exhibit high pregnancy rate during the breeding season that they are properly managed (Amundson *et al*., 2006) and uterine

contamination can be controlled especially after the resumption of the ovarian cycle (Santos *et al.*, 2009a). The current study also showed a decline in the pregnancy rate of beef cows 45.1 % (37/ 82) at 200 days post calving because several possible factors affect the reproductive performance of these cows, such as seasonal breeding, Prolonged postpartum acyclicity in suckled beef cows, nutrition, breed and uterine infection. Pregnancy rates in the day 200 after birth were more than at 150 days, the reason for that may be these cows have a longer period to overcome their reproductive problems and more chance to be pregnant. Extended post calving anestrus in suckled beef cows is one of the most common limitations to gaining a calf every year (Miller and Ungerfeld, 2008). Climatic stress, parity, extended suckling, nutritional deficiencies, and management practices were the most common reasons of prolonged calving intervals (Diskin and Kenny, 2016).

Further studies are needed to shed light on the uterine infections in beef cows and its relationship to other factors that cause weakness of the reproductive performance of these cows.

4.5 Conclusion

A moderate agreement exists among PMN %, bacteriological findings, and vaginal discharges, whereas a poor agreement exists between bacterial findings and PMN %. Results indicated that abnormal discharges do not necessarily indicate uterine infection Also, not all of the bacterial species cause inflammation and infusion of PMN to the uterine endothelium because numerous bacteria may be normal inhabitants of the uterus; endometritis after calving can also be due to other causes, such as yeast and viruses

E. coli, *S. aureus*, and calving assistance (dystocia) were the major factors affecting uterine infection in beef cows. CE and SCE insignificantly affected the reproductive performance of beef cows. Different species of pathogenic bacteria isolated from the uterus of endometritis cows were sensitive to the enrofloxacin and tetracycline than any another antibiotic used

CHAPTER 5

DETERMINATION OF SCE OCCURRENCE AND COMPARISON OF OVARIAN ACTIVITY AND ENERGY BALANCE BETWEEN POSTPARTUM BEEF AND DAIRY CATTLE

5.1 Introduction

Fertility is considered as one of the major factors of profitability in dairy farms (Galvao *et al.*, 2013). Most Holstein cows that resume ovarian activity ussually have their first follicular wave 2 weeks post calving and about 30 or 40% of these cows ovulate at week three after calving. The other 70 or 60% of these cows ovulate in the following follicular wave between 30 to 50 days postpartum. Almost 20 to 40% of cows suffer from anovulatory cases between 50 to 60 days after calving (Santos *et al*., 2009a; Galvao *et al*., 2010b). Santos *et al*. (2009a) reported in a previous study that resumption of the ovarian cyclicity earlier in lactation is always associated with optimal reproductive performance. Furthermore, another study in North America demonstrated that Holstein cows that resumed ovarian cycle 3 weeks after calving obtained high reproductive performance in comparison with other cows with delayed ovarian cycle until 50 to 60 days in lactation (Dubuc *et al*., 2012).

Many different studies have concluded that either anovulation (Galvao *et al*., 2010b) or SCE (Gilbert *et al*., 2005; Dubuc *et al*., 2010) have a negative impact on reproductive performance; causing decreased pregnancy rate per AI, and increased interval from calving to pregnancy (Gilbert *et al*., 2005). Galvao *et al*. (2010a) in their study, concluded the occurrence of SCE at day 49 was lower in the cows that resumed their ovarian cyclicity at week three postpartum in comparison with the cows that had no ovarian activity at 49 d. postpartum. Endometritis has a significant hazard in cattle (Cheong *et al*., 2011) as shown as lipopolysaccharide (LPS) level of gram-negative bacteria are elevated in the uterine fluid (Mateus *et al*., 2003) and blood (Herath *et al*., 2009) when cows develop uterine diseases. LPS has been believed to reduce the secretion of LH and GnRH and consequently result in decreased ovarian aromatase (Herath *et al*., 2009), which finally may influence follicular development, estradiol secretion (Williams *et al*., 2007), and impair ovulation rate.

A cow's body condition score represents the quantity of subcutaneous body adipose tissue (Ferguson *et al*., 1994), and is related to reproductive performance. Therefore, cows should be scored during the dry and early lactation interval to put a suitable plan to avoid negative energy balance during the postpartum period.

Negative energy balance (NEB) is considered one of the most common reasons that have an adverse effect on the resumption of ovarian activity after calving in dairy and suckled beef cows. NEB disrupts immune function, which may predispose cows

to uterine infection and subsequently reduce their ability to overcome postpartum uterine infection. The level of blood NEFA factor reflects the magnitude of fat mobilization, while the level of blood BHBA reflects the oxidation of fat in the liver (LeBlanc, 2010). A previous study by Dubuc *et al*. (2012) showed the important impacts of β-Hydroxybutyric acid (BHBA), non-esterified fatty acids (NEFA) and acute phase protein haptoglobin as an indicator of inflammation on the resumption of ovarian cyclicity during week 3 postpartum. This study found cows that had high concentrations of NEFA factor and also pre- and postpartum tend to have decreased the chance of ovarian cyclicity at 21 days postpartum. Severe NEB has negative impacts on LH frequency pulsatility, serum insulin, glucose, and IGF-1 concentrations, which causes essential impacts on follicular growth, estradiol secretion, and ovulation (Butler, 2003).

The concentration of serum IGF-1 was greater and increased more in cows that resume their ovarian function and cycled normally during three weeks after calving than the cows which have ovarian disorders such as ovarian cyst and inactive ovaries. There is a significant correlation between both estradiol hormone and IGF-1 concentration. IGF-1 is an essential factor for follicular growth (Beam and Butler, 1998; Chase *et al*., 1998). Beam and Butler (1998), also concluded that plasma IGF-1 concentration was greater approximately 40-50% in the first dominant follicle during the first 14 days after calving in dairy cows and ovulated in comparison with the cows that suffered from anovulation. Lucy *et al*. (1992) found a significant relationship between blood IGF-1 and both estrogen: progesterone hormone ratio in the ovarian follicular fluid. Other previous studies have confirmed that the concentration of IGF-1 may be influenced by energy balance after calving (Butler, 2000) and was negatively related with free fatty acid (FFA) (Nishimura *et al*., 1999). This result corresponds with a previous study that concluded a negative correlation between IGF-1 and FFA (Zulu *et al.*, 2002).

Dubuc *et al*. (2012) found in their study that both parturition season and parity affect resumption of ovarian cyclicity after calving; however, other postpartum problems like parturition disorders (e.g., dystocia, twins, stillbirths or retained placenta), lameness, mastitis, and metabolic disorders like milk fever and ketosis may directly (through inflammation), or indirectly (through NEB), influence resumption of ovarian cyclicity at early postpartum period. A previous research reported that many factors may be contributory to impairment of ovarian cyclicity and ovulation because these factors like body weight loss and length of dry period are related with NEB (Shrestha *et al*., 2005).

Prolonged postpartum anestrus in suckled beef cows is one of the main restrictions to obtaining a calf every year. According to a study by Short *et al*. (1990), prolonged calf suckling, parity, nutritional and energy deficiencies, climatic stress and management were the common reasons of prolonged calving periods. The objective of the present study is to a determination of SCE occurrence, to compare ovarian activity between healthy and SCE cows and to assess the relationship between SCE

and energy balance by evaluation of BHBA and NEFA during the postpartum period in both dairy and beef postpartum cattle.

5.2 Materials and methods

5.2.1 Animals

A total of 96 beef cows (53 Brangus and 43 Kedah-Kelantan cross breeds; 15 heifers and 81 cows) and 52 Friesian (42 cows and 10 heifers) at 20 days to 50 days post-calving period were obtained from three farms between June 2015 and May 2017. These farms are located in Serdang, Selangor where the average temperature is 28 ℃, and relative humidity is about 70%. The cows sampled were between 3 and 7 years old, weighing between 300–450 kg and managed under semi-intensive system of management. The cows were also fed with concentrated feed which was composed of alfalfa, corn silage, beet pulp, cottonseed, soybean, corn, and barley. Individual animal data on calving history, lactation, breed, and parity were all recorded. The three farms practiced natural mating.

5.2.2 Examination of animals

All cows were checked weekly by transrectal palpation and transrectal ultrasound technique from week 3 until week 16 after calving to evaluate ovarian activity.

Ovaries were scanned twice weekly using B-mode ultrasound attached with a linear probe of 7.5 MHz frequency (Sonosite VET 180 Plus, Bothell, WA, USA) from week 3 postpartum until week 16 or when the ovulation was confirmed (Fig. 5.1).The follicular diameter was measured as the mean of the two measurements. Follicles ≥ 5 mm in diameter were registered as the diameter of the largest dominant follicle at first examination. Intervals from calving to largest dominant (10 mm) follicle, and ovulation were also registered (Atkins *et al*., 2010; Gobikrushanth *et al*., 2016). An ovulation was proved when a large preovulatory follicle that was existing at last examination disappeared, leaving behind a big hole (12-15 mm) and a corpus luteum in the same location on the ovary during the following examination (Dourey *et al*., 2011). Delayed ovulation (>60 days postpartum) was used as indicators to ovarian cessation (Shrestha *et al*., 2004).

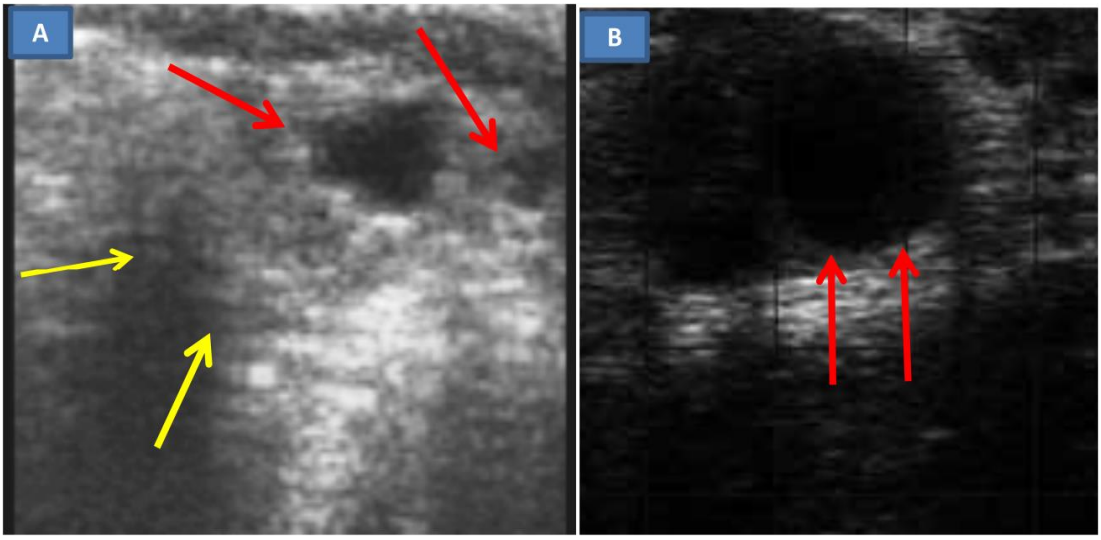

Figure 5.1 : Two growing follicles (red arrow) and yellow arrow show border of ovary (A). Red arrows indicate to preovulatory follicle (B), B-mode ultrasound with a linear probe of 7.5 MHz frequency.

5.2.3 Body condition score (BCS)

Body condition score was determined at 2 weeks before calving and at weeks 2, 4 and 6 after calving by using a 5-point scale (Ferguson *et al*., 1994).

5.2.4 Endometrial cytological sampling

Endometrial cytological samples were collected from the cows by using the modified CB as described in chapter 3(3.2.3.2).

Endometrial threshold value ≥ 8% was used (Madoz *et al*., 2013; Ricci *et al*., 2015) to determine the SCE occurrence in the farms between 20 and 30 days postpartum.

5.2.5 Blood samples

Blood samples (10 ml) were collected twice weekly from week 3 to week 7 after calving through jugular venupuncture into plain vacutainer tubes (Becton-Dickinson, NJ, USA). Blood samples were immediately placed in an ice box and transported to the laboratory within 3 h of collection. Serum was separated by centrifugation at 2500 rpm for 15 min and was transferred to 2ml microcentrifuge tubes and stored at -80 ℃ until analysis.

5.2.6 Analysis of progesterone, estradiol, IGF-1, NEFA and BHBA

Serum progesterone and estradiol-17 concentrations were measured using a commercial solid –phase radioimmunoassay (RIA) kit (progesterone, REF; IM 1188S; estrogen, REF; A 21854: IMMUNOTECH, Beckman Coulter company, France) following the manufacturers procedure. The sensitivity was 0.03 ng/mL for progesterone and 3pg/mL for estradiol. The intra- and interassay coefficients of variation were 8.3% and 15.8% for estradiol and 7.6% and 14.3% for progesterone.

The concentrations of IGF-1, β-Hydroxybutyric acid (BHBA) and nonesterified fatty acids (NEFA) were measured using an enzyme-linked immunosorbent assay (ELISA). The QAYEE® kit (Qayee-bio for life science, China) was used for IGF-1, BHBA, and the NEFA-HR(2) factor. Sensitivity was 7.8 ng/mL, 1.56 ng/mL, 7.8 ng/mL for IGF-1, NEFA and BHBA respectively. The Intra- and interassay coefficients of variation were 5.3% and 11.7% for IGF-1, and 8.2% and 13.4% for NEFA, and 7.6% and 14.5% for BHBA. All procedures were performed according to the guidelines provided by the manufacturers and methods in the literature (Kim *et al*., 2014).

5.2.7 Statistical analysis

All the statistical analyses were performed by using SPSS software (version 18.0, IBM SPSS Inc., Chicago: USA). All values (concentration of hormones) were expressed as mean ±SEM of the mean. The Shapiro–Wilk test was used to confirm the normal distribution of the traits examined. The results of the control (healthy cows) and endometritis groups were compared using the Student t-test to determine statistical significance. Statistically differences among samples collected on weeks 3, 4, 5, 6 and 7 postpartum in the two groups were calculated using one-way ANOVA as well as Tukey and Duncan post hoc tests at the probability threshold, $P < 0.05$. Since the data on resumption of ovarian activity was non-parametric data, we used Mann-Whitney test was used to analyze the results among endometritis, healthy, heifers and multiparous cows groups.

5.3 Results

5.3.1 Occurrence of SCE

The overall occurrence of SCE in beef and dairy cows was 13.5 % (20/148) at week 4 postpartum. The results of endometrial cytology examination at week 4 (21-28 days postpartum) for beef group showed that a total of 96 beef animals, 12.5% (12/96), heifers 5.2% (5/96) and multiparous 7.3% (7/96) were positive to SCE (≥ 8 PMN %). Out of the total 15 beef heifers, 33.3% (5/15) were positive to SCE which was significantly ($P < 0.05$) difference than multiparous beef cows 8.6 % (7/81) (Table 5.1). The results revealed 8/52 cows (15.3 %) from dairy group, heifers 7.6 %

(4/52), and multiparous cows 7.6% (4/52) had SCE. Out of the total 10 dairy heifers, there were 40% (4/10) positive to SCE ($P < 0.05$) than multiparous dairy cows 9.5% (4/42) (Table 5.1).

Table 5.1 : SCE occurrence in heifers and multiparous dairy and beef groups.

Animals	Dairy group SCE (%)	Beef group SCE (%)
Heifers	4/10 (40%) [a]	5/15 (33.3%) [a]
Multiparous	4/42 (9.5%) [b]	7/81 (8.6%) [b]
Total	8/52 (15.3%)	12/96 (12.5%)

[ab] Means values within columns between heifers and multiparous with different superscripts indicate a significant difference ($P < 0.05$).

Also, the study showed there was a significant difference among breed groups in the occurrence of SCE, K.K breed 4.6% (2/43) was significantly lowest ($P < 0.05$) than Friesian dairy cows 15.3% (8/52), and Brangus beef 18.8 % (10/53) (Table 5.2).

Table 5.2 : SCE occurrence in different breeds at week 4 postpartum.

Breed	Total	SCE (%)
Friesian cross breed	52	8 (15.4%) [a]
Brangus	53	10 (18.9%) [a]
K.K cross breed	43	2 (4.7%) [b]
Total	148	20 (13.5%)

[ab] Means values within column among breeds with different superscripts indicate a significant difference ($P < 0.05$).

5.3.2 Transrectal palpation and ultrasound examination results

The study showed that out of a total 148 beef and dairy animals (96 beef and 52 dairy groups), 64.9 % (96/148) had cessation of ovarian cyclicity during the first 60 days postpartum period (Table 5.3), percentage of delayed resumption of ovarian activity was significantly lower 27.3% in healthy than SCE dairy group 75.0%. There was no significant difference between healthy and SCE beef groups, 80.9 % and 83.3% respectively (Table 5.3). The cessation of ovarian cycle was lower in Friesian group, 18 /52 (34.6%) ($P < 0.05$) than K.K, 32 /43 (74.5%) and Brangus beef group, 46/53 (86.8%) during 60 days postpartum (Table 5.4).

Table 5.3 : Occurrence of delayed resumption of ovarian activity in healthy and SCE dairy and beef groups.

Animals	Status	Total	Delayed resumption of ovarian activity (%)
Dairy group	Healthy	44	12 (27.3%) [a]
	SCE	8	6 (75.0%) [b]
Beef group	Healthy	84	68 (80.9%) [b]
	SCE	12	10 (83.3%) [b]
Total		148	96 (64.9%)

[ab] Means values within column among groups with different superscripts indicate a significant difference ($P < 0.05$).

Table 5.4 : Breed differences in resumption of ovarian activity of cows.

Group		Total	Acycling cow	Cycling cows (Ovarian Resumption)		
				< 30 days)	30-50 days	51- 60 days
Dairy group		52	18 (34.6%) [a]	7(13.5%)	9 (17.3%) [a]	18 (34.6%) [a]
Beef group	KK	43	32 (74.5%) [b]	0	2 (4.6. %) [b]	9 (20.9%) [b]
	Brangus	53	46 (86.8%) [b]	0	0 (0 %) [c]	7 (13.2%) [b]

[abc] Means values within columns among groups with different superscripts indicate significant difference ($P < 0.05$).

In the present study, a proportion of dairy animals that ovulated before 30 days postpartum was13.5% (7/52), while 17% (9/52) were ovulated between 30 and 50 days postpartum and it was significantly higher than KK and Brangus beef groups. Also, in the period between 50 and 60 days after calving, the ovarian cyclicity was significantly higher in dairy group 34.6 % (18/52) than KK (20.9%) and Brangus (13.2%) beef groups (Table 5.4)

Out of total 15 beef heifers, 73.3% (11/15) had ovarian cessation compared with multiparous beef cows 82.7% (67/81) and without significant difference. The results in dairy group revealed that out of 10 dairy heifers 80% (8/10) suffered from cessation of ovarian activity significantly higher than multiparous dairy cows 23.8% (10/42) (Table 5.5)

Table 5.5 : Delayed resumption of ovarian activity in heifers and multiparous dairy and beef group.

Animals	Delayed resumption of ovarian activity (%)	Delayed resumption of ovarian activity (%)
	Dairy group	Beef group
Heifers	8/10 (80%) [a]	11/15 (73.3%) [a]
Multiparous	10/42 (23.8%) [b]	67/81 (82.7%) [a]
Total	18/52 (34.6%)	78/96 (81.2%)

[ab] Means values within columns between heifers and multiparous with different superscripts indicate a significant difference ($P < 0.05$).

The resumption of ovarian activity (60 days postpartum) was higher in healthy group in dairy and beef animals compared with SCE group, with significant difference ($P < 0.05$) only in the dairy group (Table 5.6). Also, the resumption of ovarian activity was greater in multiparous in dairy and beef cows than heifers which was significant ($P < 0.05$) for the dairy group (Table 5.6).

Table 5.6 : Effect of health, parity and BCS status on ovarian resumption in dairy and beef groups.

Variable	Ovarian resumption (%)	Ovarian resumption (%)
	Dairy group	Beef group
Health		
SCE	2/8 (25 %) [a]	2/12 (16.7 %) [a]
Healthy	32/44(72.7 %) [b]	16/84(19 %) [a]
Parity		
multiparious	32/42 (76.2 %) [a]	14/81 (17.3) [a]
heifers	2/10 (20 %) [b]	4/15 (26.7 %) [a]
BCS		
< 2.5	8/24 (33.3 %) [a]	4/51 (7.8 %) [a]
≥ 2.5	26/28 (92.9 %) [b]	14/45 (31.1 %) [b]

[ab] Means values along columns within each variable with different superscripts indicate a significant difference ($P < 0.05$).

The mean diameter of large follicle (9.3± 0.6 mm) was significantly higher in healthy dairy group than cows that had SCE (5.2 ± 0.3 mm) at week 4 and 5 postpartum (Table 5.7). The mean interval time from calving to ovulation in healthy dairy group (29.4 ± 0.7days) was faster ($P < 0.05$) compared with SCE group (47.5± 0.9 days) (Table 5.7)

The ovarian function of beef group at week 3 postpartum was weak in both SCE and healthy groups, and the size of follicles was less than 5mm especially in SCE group. The mean interval time from calving to ovulation was faster in healthy beef group 50.3 ±0.7 days than SCE group 57.6 ±0.5 postpartum (Table 5.7).

Table 5.7 : Largest dominant follicle, first appearance of dominant follicle and first ovulation interval calving (Mean±SEM) between healthy and SCE dairy and beef cows.

Variable	Dairy cows		Beef cows	
	Healthy	SCE	Healthy	SCE
DLF (mm)	9.3 ± 0.6 [a]	5.2 ± 0.3 [b]	5.4 ± 0.3 [a]	4.8 ± 0.7 [a]
ICDF (days)	20.5 ±0.9 [a]	37.1 ± 0.7 [b]	43.6 ±0.4 [a]	48.7 ±0.1 [a]
ICFO (days)	29.4 ± 0.7 [a]	47.5 ± 0.9 [b]	50.3 ±0.7 [a]	57.6 ±0.5 [a]

DLF: diameter of the largest follicle at first examination; ICDF, interval from calving to the first appearance of a dominant follicle (10 mm in diameter); ICFO, mean (±SE) interval from calving to first ovulation. [ab] Means values with different superscripts within rows indicate a significantly difference ($P < 0.05$).

5.3.3 Body condition score (BCS)

Generally, BCS was higher in healthy, cycling dairy and beef groups compared with SCE and acycling groups. In dairy animals, There was no significant difference between healthy 3.25 ± 0.09 and SCE group 3.1 ±0.08 at 14 days before calving; and at calving 3 ± 0.1, 2.9 ± 0.09 respectively. There was a significant difference ($P < 0.05$) at week 2 and 4 postpartum between BCS healthy (2.8 ± 0.04, 2.7 ± 0.045) and SCE groups (2.5 ± 0.09, 2.4 ± 0.05) respectively. The present study did not find any significant difference between both healthy dairy and SCE cows at week 6 after calving (Fig 5.2).

The results did not show any significant difference between both healthy (3.3 ± 0.08, 3.1 ± 0.04) and SCE beef groups (3.2 ± 0.07, 3 ± 0.03) before two weeks of calving and at calving respectively. There was a significant difference between healthy (2.8 ± 0.05) and SCE (2.6 ± 0.03) animals at week 2 after calving while the results findings did not find any difference after week 4 and 6 postpartum (Fig 5.3).

The present study reported a significant difference ($P < 0.05$) in BCS between both cycling and acycling in the dairy (Week 2, 4 and 6) and beef animals (week 4 and 6) postpartum (Fig 5.4, 5.5). The resumption of ovarian activity was greater (92.9%) and (31.1%) in both dairy and beef groups respectively in cows that had BCS >2.5 compared with animals had ≤ 2.5 during the postpartum period (Table 5.6).

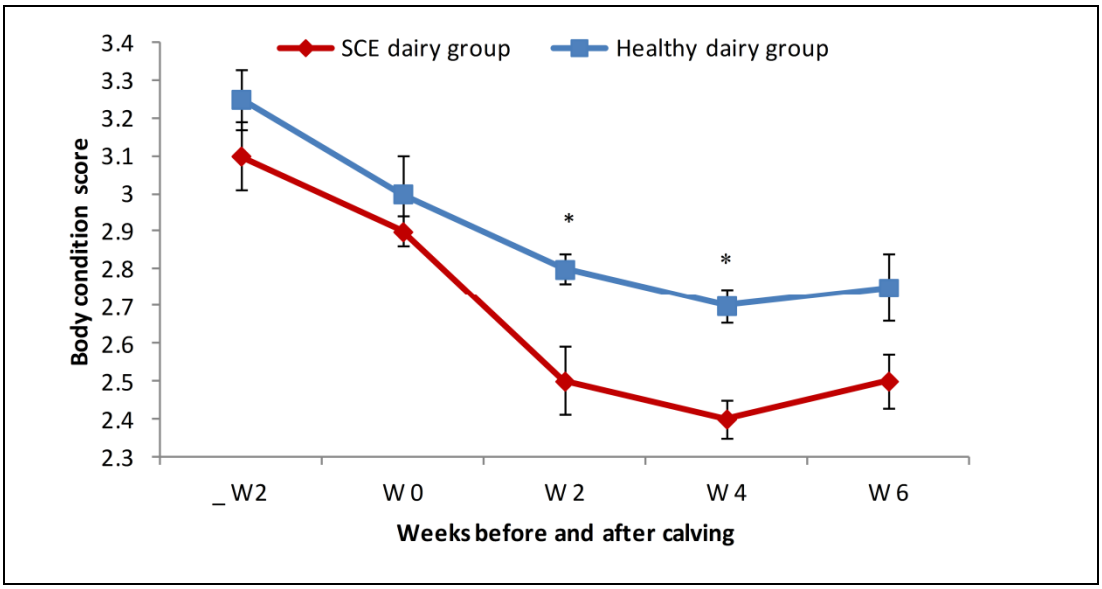

Figure 5.2 : BCS in healthy and SEC dairy group in prepartum and postpartum period.
* indicate significant difference at P < 0.05.

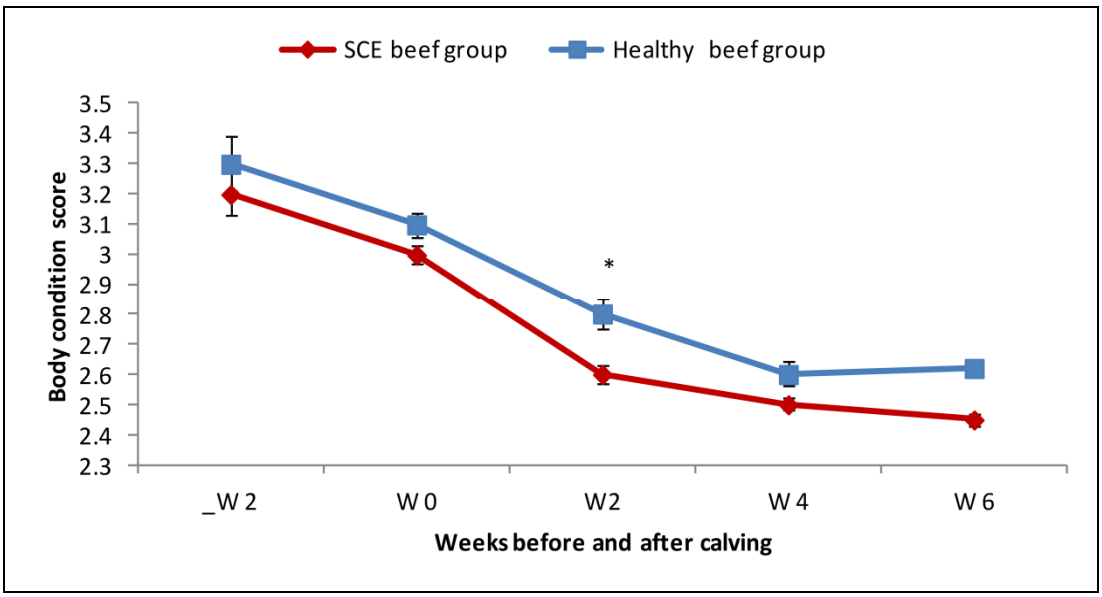

Figure 5.3 : BCS in healthy and SCE beef group in prepartum and postpartum period.
* indicate significant difference at P < 0.05.

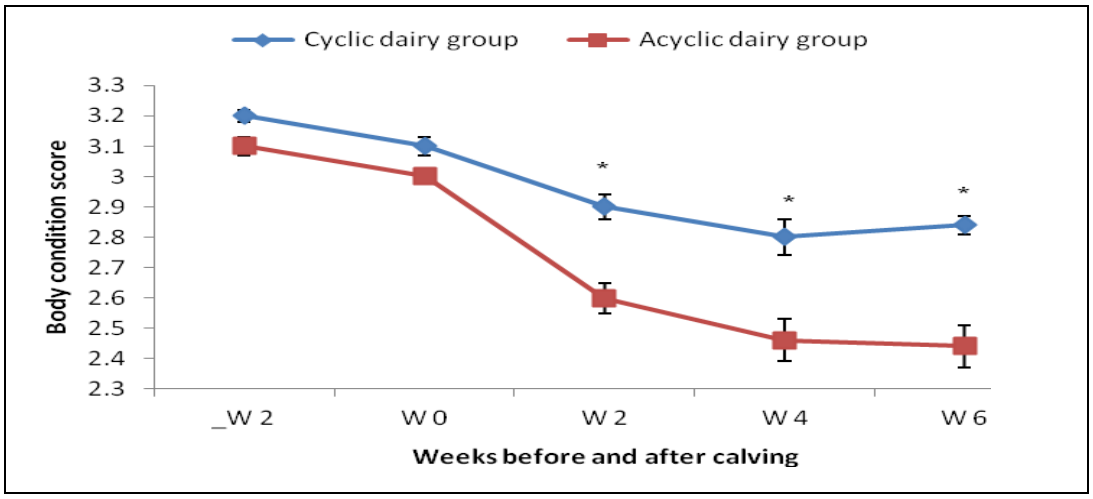

Figure 5.4 : BCS in cyclic and acyclic dairy group in prepartum and postpartum period.
* indicate significant difference at P < 0.05.

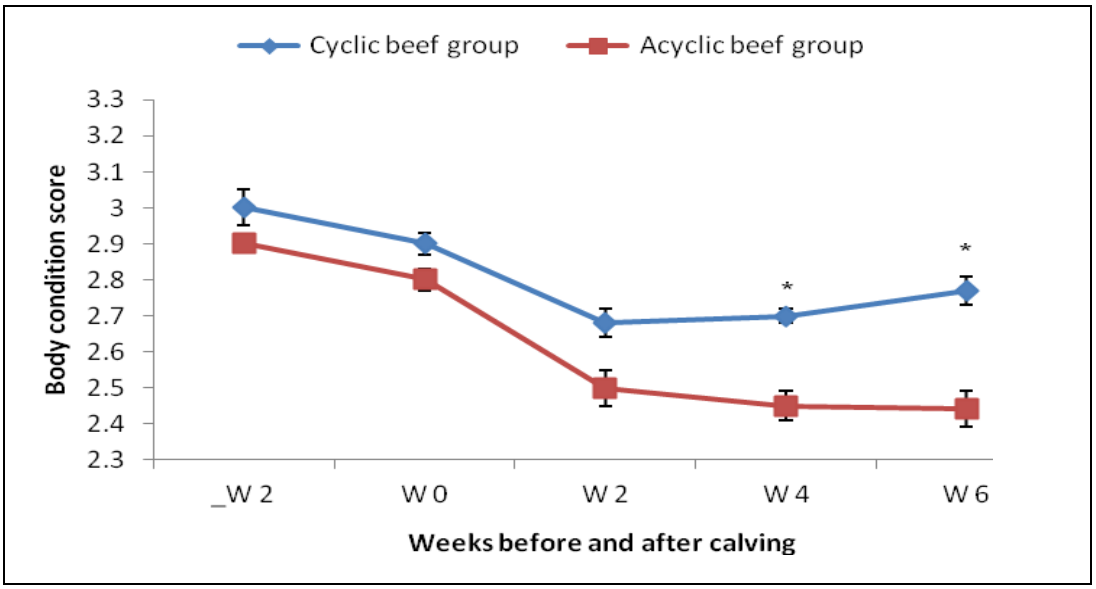

Figure 5.5 : BCS in cyclic and acyclic beef group in prepartum and postpartum period.
* indicate significant difference at P < 0.05.

5.3.4 IGF-1, Progesterone, Oestradiol, NEFA and BHBA concentrations

As shown in Fig. 5.6 and 5.7, serum IGF-1 concentration in both dairy and beef cattle were higher in healthy group compared with the SCE group showing a significant difference (P < 0.05) at weeks 3 and 5 in the dairy group. Serum IGF-1 concentration in dairy cows was slightly higher than beef cows and either group

showed progressive increase in serum IGF-1 concentration with the advance of the postpartum period. Progesterone concentration was less than 1 ng/mL in both SCE and healthy beef cows, the progesterone profile was higher slightly in healthy cows than SCE group across week 3 until week 7 after calving with significant difference ($P < 0.05$) at week 4 and week 6 postpartum (Fig. 5.8). In the dairy group, the progesterone level was higher for the most weeks of the study in healthy cow compared with SCE group (Fig. 5.9). Progesterone concentration increased gradually in healthy cows at day 30 and reached peak >3 ng/ mL at 47 days postpartum compared with SCE group 1ng/mL at 44 days postpartum.

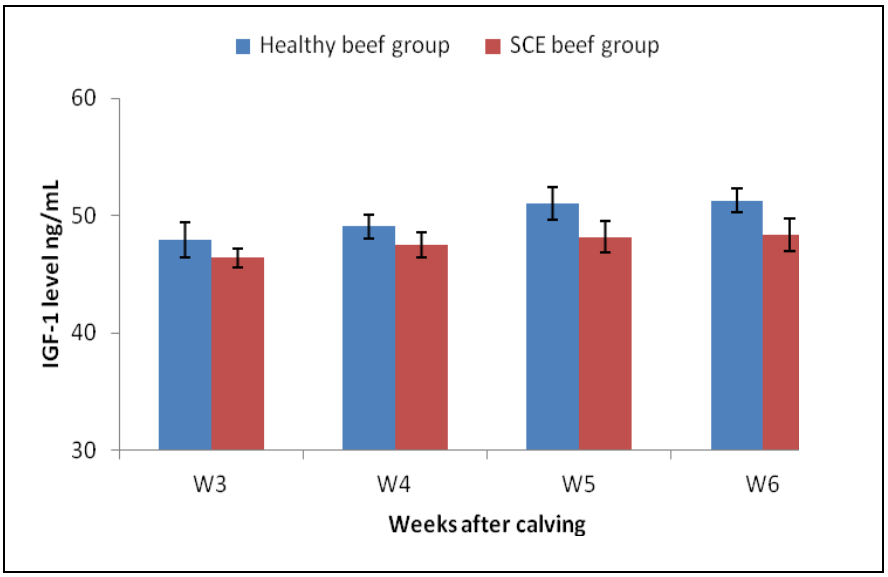

Figure 5.6 : Serum IGF-1 levels Mean ±SEM in healthy (n=12) and SCE (n=12) beef group.
in postpartum period. There are no significant between the two groups.

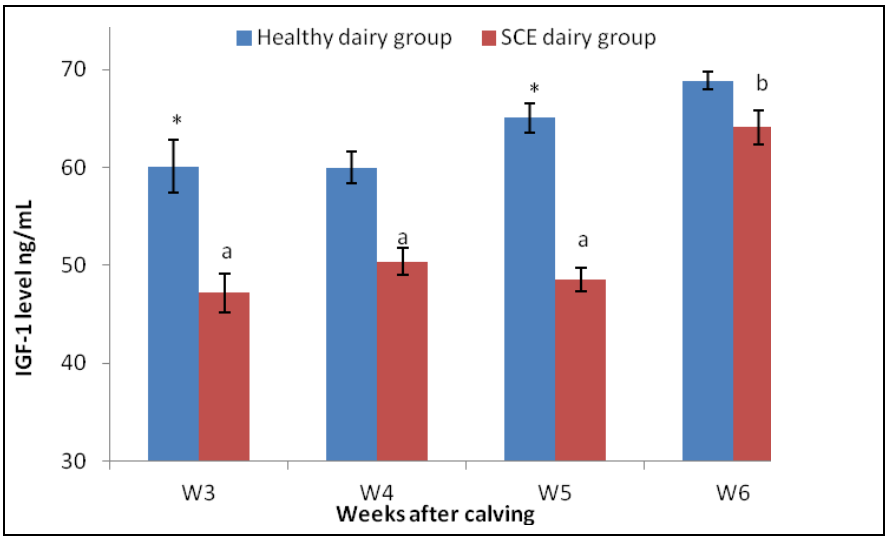

Figure 5.7 : Serum IGF-1 level Mean ±SEM in healthy (n=8) and SCE (n=8) dairy groups in postpartum period. * indicate significant difference at P < 0.05 between healthy and SCE groups.

[ab] Means values among weeks in same group with different superscripts indicate significant difference at P < 0.05.

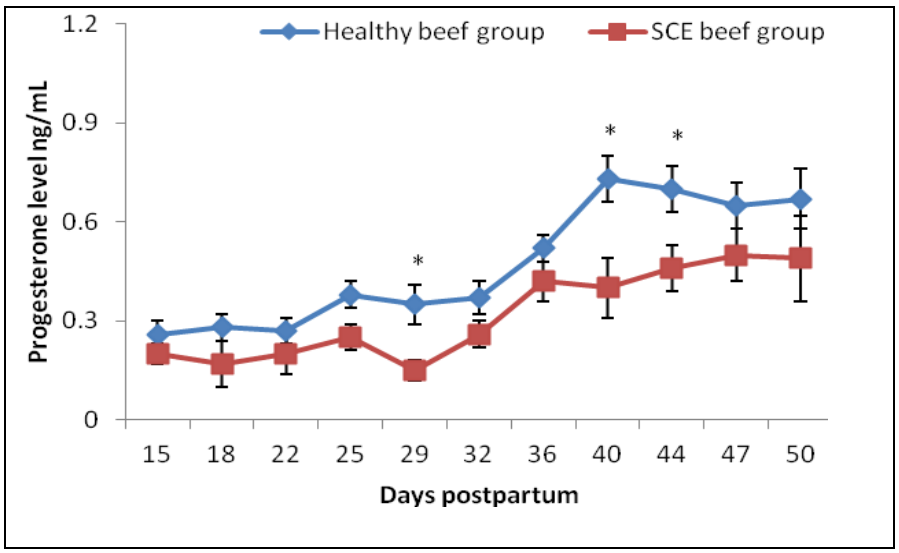

Figure 5.8 : Serum progesterone profile Mean ±SEM in both healthy and SCE beef groups.

* indicated significant difference at P < 0.05 between healthy and SCE group.

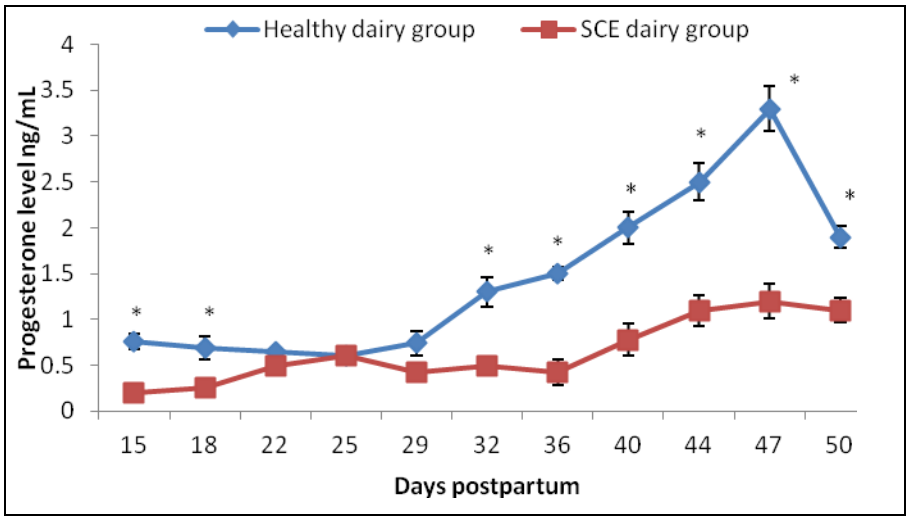

Figure 5.9 : Serum progesterone profile Mean ±SEM in both healthy and SCE dairy groups.
* indicate significant difference at P < 0.05 between healthy and SCE groups.

Estradiol levels were significantly (P < 0.05) higher at weeks 4 (6.8 ± 1.1 pg/mL) and 6 (9.8 ± 1.3 pg/mL) in healthy group than SCE one after calving in the beef group (Fig 5.10). In dairy cows, concentrations of estradiol were higher (P < 0.05) in healthy cows at weeks 3, 4 and 5 (11.7 ± 1.2, 12.5 ± 1.1, 16.7 ± 1.6 pg/mL) compared with cows suffered from SCE (5.1± 1, 6.3 ± 0.9, 5.2 ± 1.3 pg/mL) respectively. There was no significant (P > 0.05) difference of estradiol levels in both healthy and SCE at weeks 6 and 7 postpartum (Fig 5.11).

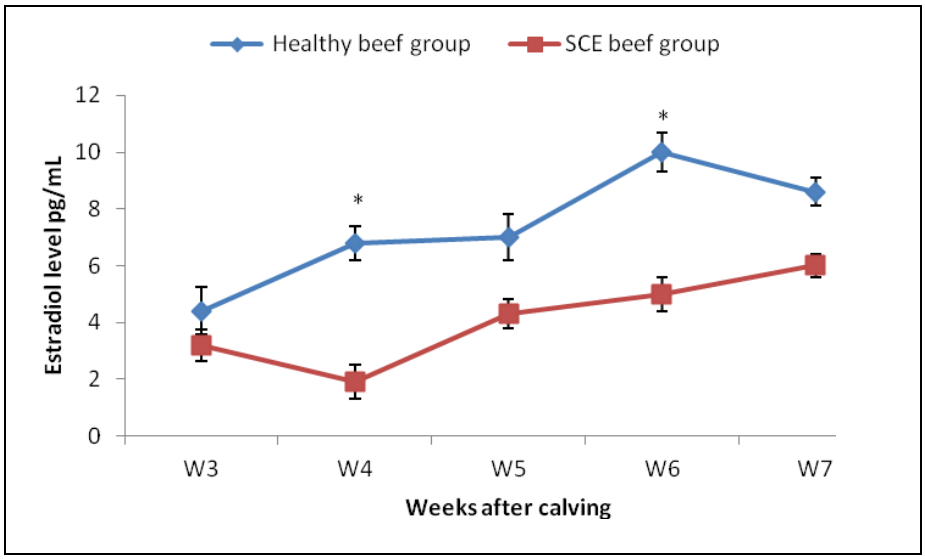

Figure 5.10 : Serum estradiol profile Mean ±SEM in both healthy and SCE beef postpartum groups.
* indicate significant difference at P < 0.05 between healthy and SCE groups.

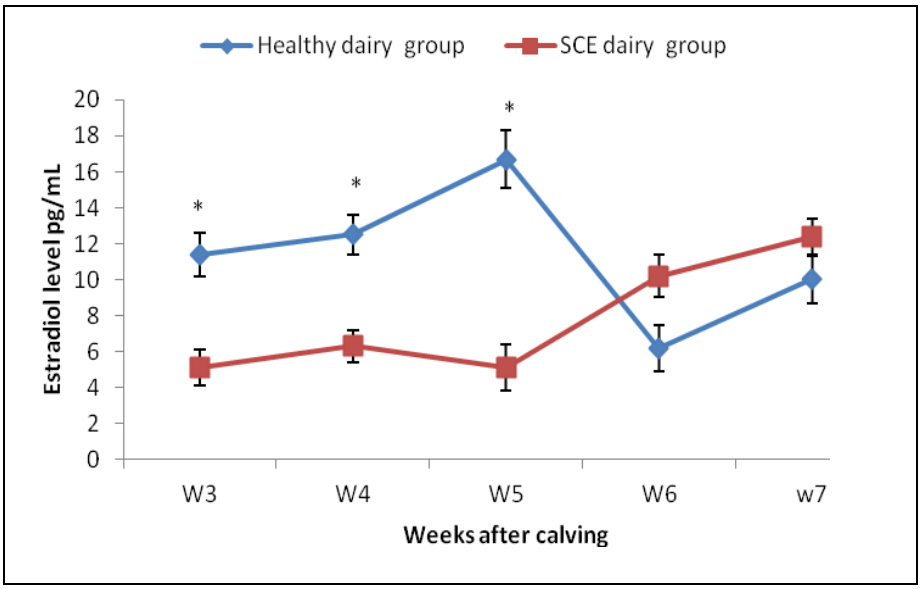

Figure 5.11 : Serum estradiol profile Mean ±SEM in both healthy and SCE dairy postpartum groups.
* indicate significant difference at p< 0.05 between healthy and SCE groups.

The level of serum NEFA in SCE beef group was significantly higher at weeks 3 and 4 postpartum (78.4 ± 2.1, 77.6 ± 3 ng/ mL) compared with healthy group (62.1 ± 1.1, 63.2 ± 1.2 ng/ mL) respectively. Also, the concentration of NEFA at weeks 5 and 6 postpartum were higher in SCE than a healthy group but without significant ($P > 0.05$) difference (Fig 5.12). Serum NEFA at weeks 5 and 6 were significantly ($P < 0.05$) lower than weeks 3 and 4 postpartum in SCE beef group.

In dairy group, the level of NEFA in SCE group was significantly higher at weeks 3, 4 and 5 postpartum (77. 2 ± 5.1, 73.2 ± 5.6 and 72 ± 2.3 ng/ mL) compared with healthy animals (62.1 ± 2.1, 60.1 ± 0.8 and 61.2 ± 2.5 ng/ mL) respectively. There was no significant ($P > 0.05$) difference at week 6 between healthy and SCE groups (Fig 5.13). The study showed significantly decreasing in Serum NEFA at week 6 postpartum compared with the previous weeks in SCE dairy group.

The BHBA concentration was significantly ($P < 0.05$) higher in SCE beef and dairy groups at weeks 3, 4 after calving (390.3 ± 23.1, 374.8 ± 22.7 ng/ mL), (334.6 ± 11.2, 320.6 ± 13.4 ng/ mL) respectively compared with healthy beef and dairy group (310.2 ± 8.4, 299.4 ± 10.8 ng/ mL), (266.9 ± 10.2, 268.9 ± 6.4 ng/ mL) respectively (Fig 5.14 and 5.15). Although the concentration of BHBA was higher in SCE beef and dairy groups at weeks 5 and 6 after calving, it was not significant ($P > 0.05$) compared with healthy groups.

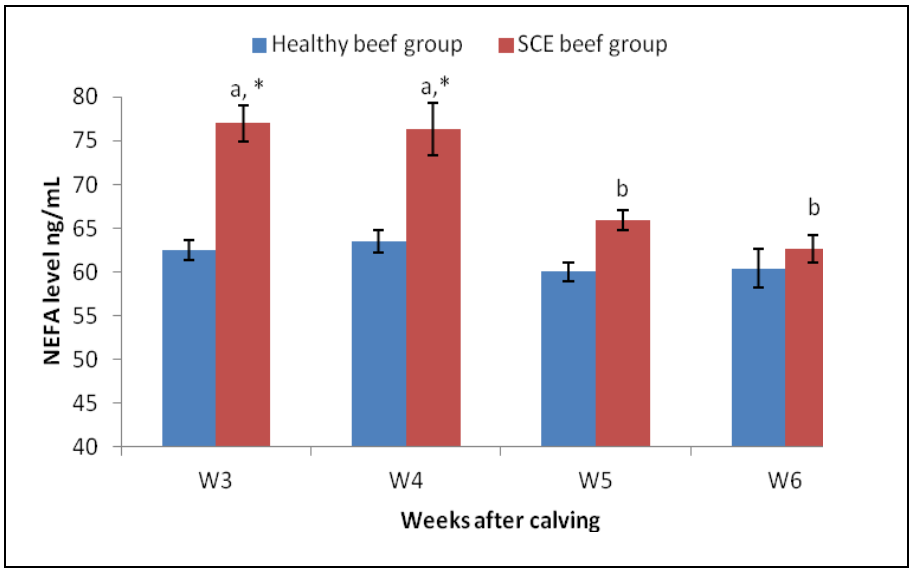

Figure 5.12 : Serum NEFA levels Mean ±SEM in both healthy (n=12) and SCE (n=12) beef groups.
* indicate significant difference at p< 0.05 between healthy and SCE groups.
[ab] Means values among weeks in same group with different superscripts indicate significant difference at P < 0.05.

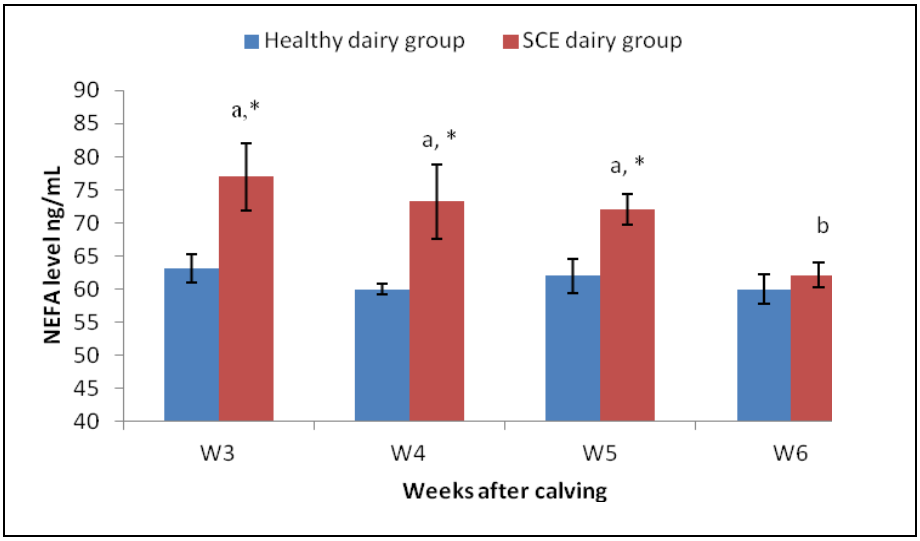

Figure 5.13 : Serum NEFA levels Mean ±SEM in both healthy (n=8) and SCE (n=8) dairy groups.
* indicate significant difference at p< 0.05 between healthy and SCE groups.
[ab] Means values among weeks in same group with different superscripts indicate significant difference at P < 0.05.

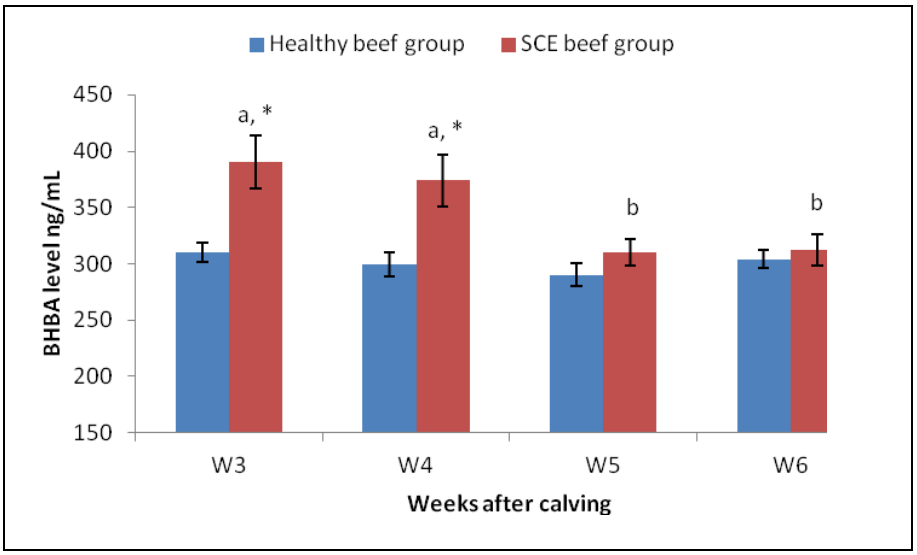

Figure 5.14 : Serum BHBA levels Mean ±SEM in both healthy (n=12) and SCE (n=12) beef groups.
* indicate significant difference at p< 0.05 between healthy and SCE groups.
ab Means values among weeks in same group with different superscripts indicate significant difference at P < 0.05.

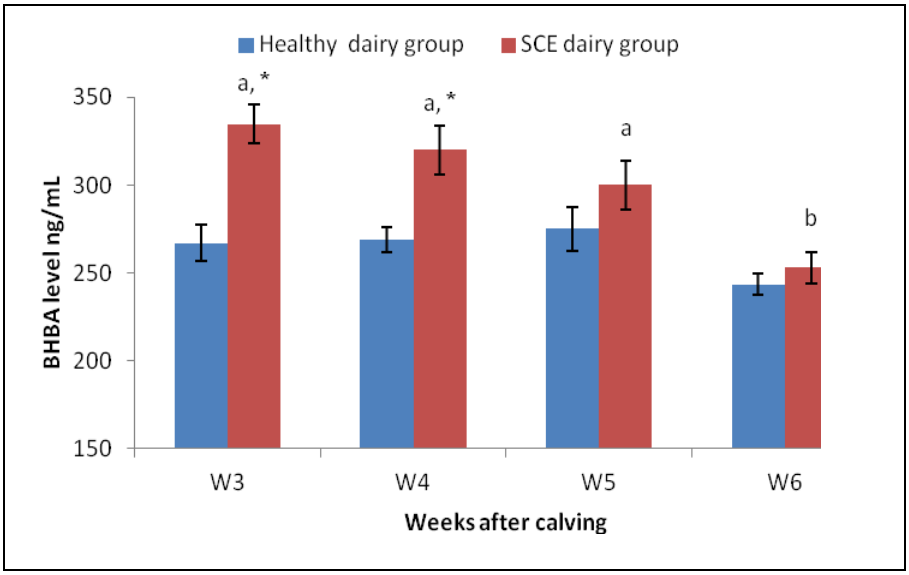

Figure 5.15 : Serum BHBA levels Mean ±SEM, in both healthy (n=8) and SCE (n=8) dairy groups.
* indicate to significant difference at p< 0.05 between healthy and SCE groups.
ab Means values among weeks in same group with different superscripts indicate significant
difference at P < 0.05.

5.4 Discussion

Most of the sampled cows suffer postpartum disorders within 30 days after parturition (LeBlanc *et al*., 2006). There are many disorders and diseases like dystocia and uterine infection related to the postpartum period (McDougall, 2001; Sheldon *et al*., 2009). Other common risk factors that may predispose cows to developing postpartum diseases includes impairment of postpartum immune activity (Hammon *et al*., 2006), hormonal and metabolic alteration (Bauman and Currie, 1980; Yimer, 2011), decrease in dry matter intake (DMI) dietary imbalances (Drackley, 1999) and unsuitable environment (LeBlanc *et al*., 2006). Therefore, minimization of these risk factors is important for increasing the reproductive performance of these cows.

5.4.1 SCE occurrence in beef and dairy group

The present study showed that occurrence of SCE in beef cows was 12.5 % and about 15.3 % in dairy cows and these results were lower than previous studies for both breeds of cows (Kasimanickam *et al*., 2004; Gilbert *et al*., 2005; Santos *et al*., 2009b). There were only two studies (Santos *et al*., 2009b; Ricci et al., 2015) that have reported SCE in beef cows. Santos *et al*. (2009b) reported in a previous study in beef cows that 17% of Angus cows (2-78 d postpartum) were positive to endometritis by using low-volume uterine lavage method which is greater than our findings in beef cows and this may be because this study used threshold of 5.5% PMN as indicator for SCE and used LVF method to collect endometrial cytology. This study found the ability of beef cows in the postpartum period to clear uterine infection after the resumption of ovarian activity without impairment of fertility (Santos *et al*., 2009b). In contrast, the second survey for Ricci *et al*. (2015) included 97 beef cows between 28 and 68 days postpartum were sampled; this study demonstrated a 31% prevalence of SCE with reduction of reproductive performance.

The occurrence of SCE in dairy group in the present study was 15.3%, which is greater than a report of 11.8% by Barlund *et al*. (2008), who used 8% PMN as the threshold during 28 to 41 postpartum. However, this occurrence was lower than in another study (Denis Robichaud and Dubuc 2015) where it was reported 36% by using a threshold of 6% PMN at 28 to 42 days after calving. The same survey reported the increased percentage of SCE (48%) when the diagnosis was depended leukocyte esterase technique (Denis Robichaud and Dubuc 2015). Thus far, no consensus has been established with regard to the effect of threshold value and time of uterine sampling on SCE diagnosis. SCE can be diagnosed using different cut-off values, such as PMN range of 5–18%with either cytobrush or low-volume lavage techniques (Kasmanickam *et al*., 2004; Gilbert *et al*., 2005; Barlund *et al*., 2008). Other studies depended on the thresholds of PMN % according to the effects on the reproductive performance (Cheong *et al*., 2011; McDougall *et al*., 2011). Kasmanickam *et al*. (2004) depended on >18% PMNs as threshold value between 20 -33 days postpartum and >10% PMNs between 34 and 47 days postpartum using

cytobrush technique to diagnosis SCE while Gilbert *et al*. (2005) used >5% PMNs as a significant cut-off point for diagnosis endometritis in cows using lavage method between 40 and 60 days postpartum. The low SCE prevalence may be attributed to differences in geographic area, environment, and the number of endometrial cells counted among the studies. A total of 300 cells were counted per slide in the present study, whereas 100 cells were counted in previous studies (Barlund *et al*., 2008).

The occurrence of SCE was little higher in dairy group than beef cows. This may be due to milk production stress factor for these cows in comparison with beef cows. This agrees with previous studies that reported lower SCE cases in beef cows and considered it a minor problem in beef farms and got 90 % of pregnancy rate with optimal management (Santos *et al*., 2009b). The findings also showed that even in beef cows, prevalence of SCE was higher in Brangus breed (18.8%) than K.K cows (4.6%) and this could be due to a genetic difference between the two groups of cows. In addition, the differences could be due to the adaptation of the local breed, the K.K. cows to the hot and humid tropical environment (Perea-Ganchou *et al*., 2005) as compared with the imported Brangus breed. The study also showed increasing SCE in heifers than multiparous in two dairy and beef groups and this may be due tocalving problems and postpartum disorders as they were more in heifers than multiparous cows.

5.4.2 Resumption of ovarian activity

The duration of estrus and its absence after calving has an essential effect on fertility (Lucy, 2006). Opsomer *et al*. (2000) concluded increasing the occurrence of anestrus in high-production dairy farms. Perhaps increase in the demand for energy to milk yielding can cause a delay in ovarian resumption post calving. However, there are several factors like low body energy reserve, postpartum disorder and restricted energy intake could also cause postpartum anestrus. Maintaining the health of cows during the periparturient period is the best way to reduce anestrus cases in dairy farms (Peter *et al*., 2009).

Most of the SCE group in dairy and beef cattle suffer from the delayed resumption of ovarian function and this agrees with studies by Senosy *et al*. (2009) and also Burke *et al*. (2010) who demonstrated a link between uterine infection and ovarian function during 5 to 6 wk postpartum, and the effects of endometritis on anestrus occurrence after 40 days postpartum. This agrees with previous studies that confirmed the effect of uterine infection on the ovarian activity (Williams *et al*., 2007). The healthy dairy group in the present study showed the significantly lower of ovarian cessation compared with healthy beef animals and SCE group in both dairy and beef groups. This result can be explained due to the ability of the healthy group to get rid of most uterine contamination and resumption of ovarian activity earlier than other groups

In the present study, the occurrence of cessation of cycling in dairy cows was 34.6 %. This agrees with a previous study which reported 38% of anestrus occurring during 50 to 60 days postpartum (Rhodes *et al*., 2003). Our finding (34.6 %) of ovarian cessation in the dairy group was higher compared with another study by Yimer (2011) who found 6.1% of dairy cows suffered from ovarian cessation in Friesian cows. The same study showed a higher cessation of ovarian activity occurred in Brangus beef cows (41%) in comparison with K.K breed (Yimer, 2011) and these were found to be lower than results in this study. This observed difference could partly be explained by the fact that samples collected during this study was during early postpartum period (week 3 and 7 postpartum) while the previous studies was achieved based on cows with fertility problems. The results of the present study also agrees with other earlier results, which included a report where 40% of cows where not cycling (Bartlett *et al*., 1987; Opsomer, 1999). Fischer *et al.* (1998) also showed a greater prevalence (49%) of anoestrus after calving. Our results disagree with another study which reported lower prevalence of anestrus (16 to 20%) in postpartum dairy cows (Mayer *et al*., 1987). The variation in the prevalence of anestrus among different studies may be because of dissimilar environmental, management conditions as well as differences in energy intake levels at different farms (Bostedt *et al*., 1985; Mwaanga & Janowski, 2000; Opsomer and kruif, 1999).

Cows with postpartum disorders could still have 30-60% ovarian inactivity (Munro et al., 1982). This difference may be due to differences in management condition, individual cow reproductive status or may be due to the use of different diagnostic techniques (Mwaanga and Janowski, 2000; Opsomer and kruif, 1999; Zduńczyk *et al*., 1992). The average cessation of ovarian activity in this study was higher in beef group (81.3%) than dairy cows (34.6%) and most of these cows suffered from inactive ovaries and were having low (1ng/mL) serum progesterone values during 7 weeks of the postpartum period. This may be due to the effect of prolonged suckling in beef cows Another reason can be explained by lesser stress on dairy cows in present study as shown by their low milk production (5-10 kg daily) (yavas and Walton, 2000) compared with the cows of high milk production (30 kg) in commercial milk production farms.

One of the main reasons for the reduction in reproductive performance in suckling beef cows is a prolonged anestrous period after calving and may exceed 120 days (Quintans and Vázquez, 2002). The duration of calving to first estrus interval was greater for beef cows calving with thin body condition. Both postpartum protein intake and body condition score at calving have been found to influence the size of the dominant follicle at the first postpartum estrus (Lents *et al*., 2008).

The cessation of ovarian activity in the present study was higher in heifers (80%) compared with multiparous (23.8%) dairy cows and this result corresponds with other studies (Opsomer *et al*., 2000; Santos *et al*., 2009a**;** López *et al*., 2012). This could be due to the fact that most of the heifers suffer from calving complications and postpartum disorders compared with healthy multiparous cows that overcome

their postpartum problems and resume ovarian cyclicity some few weeks after calving.

Size of ovulatory follicle is considered one of the most common essential factors that affect the reproductive performances in cows. A follicle having a diameter of 10 mm is considered as an ovulatory follicle in dairy cows (Sartori *et al*., 2001). Healthy cows sampled in this study at 4 week postpartum had follicular size of 9mm, and those at week 5 postpartum had follicular size of 10-12 mm. However, in cows suffering from SCE at four to five weeks postpartum, follicular size was found to be less than 6mm this agrees with previous studies that confirmed effect of uterine infection on the ovarian activity (Williams *et al*., 2007).

Furthermore, healthy beef cows in this study had larger follicle diameter than SCE cows during most weeks of this study. However, most of these follicles in the early postpartum period were small in size (less than 5 mm) and became atretic follicles, thus making most of the beef cows being in anoestrus (81.3%). A higher proportion of cows that suffer from uterine infection are often characterized by follicles having small sized diameter and most usually become atretic follicles or become small corpus luteum, after ovulation and will only secrete little amounts of the progesterone. This finding correspond with a previous study of Dourey *et al*. (2011) who found that the mean period from parturition until first ovulation was greater (45 days) in the cows that had endometritis (>8% PMN) compared with healthy cows (32 days) that have (<8% PMN) endometrial cytology.

The present study showed period from calving to the first appearance of dominant follicle and ovulation was shorter in healthy in both dairy and beef cows than SCE group. Meanwhile, our results disagree with a recent study by Gobikrushanuth *et al*. (2016) that revealed that the period from parturition until the ovary develops dominant follicles was not related with endometritis cases in cows. Although the evidence showed the effect that uterine infection might have on the LH frequency pulse, the interval from calving to the time of development of an ovulatory follicle did not differ between endometritis and healthy cows. Moreover, the period from parturition to first ovulation was between 21 and 28 days after calving in healthy dairy cows (Colazo *et al.,* 2009) and this was not different in cows that were diagnosed with endometritis which occurred at 25 days after calving.

The present results are also not in accordance with another study by Sheldon *et al*. (2002), who found that the period from parturition to first ovulation was not related and affected by bacterial contamination at many different postpartum periods. The difference among studies may be due to the difference of the type and intensity of uterine infection, cows breed, parity, environment and geographic area on ovarian activity in the postpartum period.

Uterine diseases at early postpartum period inhibit ovarian granulosa cell activity as well as the growth of dominant follicles (Williams *et al*., 2007) and this causes a suppression in synthesis and release of estradiol resulting in change in follicle lifespan and ovulation (Herath *et al*., 2007). In addition, uterine diseases also inhibit LH secretion and affect ovarian activity, which further disrupts ovulation processes in cows that have endometritis (Sheldon *et al*., 2002). Unfortunately, the present study did not involve LH hormone determination or its effect on ovarian activity in postpartum cows.

The present study showed a delay of resumption of ovarian activity in postpartum cows especially in beef and dairy cows with SCE. The transrectal palpation and ultrasound examination of the ovaries of these cows revealed follicles with small size diameters usually less than 5 mm during the early postpartum period until week 6 postpartum when the diameter of the follicles begins to increase in size. These results agree with many previous studies that have demonstrated the effect of uterine diseases after calving on ovarian resumption and dominant follicle development and formation of functional CL (Sheldon *et al*., 2002; Williams *et al*., 2007).

Although the prevalence of SCE was not so high, most of the beef group of the current study showed the absence of estrus activity (81.3 %). Although, there is a significant relationship between endometritis and ovarian disorder, another important factor that helps to clarify the association between uterine infection and ovarian disorder. The fact that both are positively related to the different degrees of negative energy balance after calving (Hammon *et al*., 2006; Galvao *et al*., 2010a).

This explanation agrees with our clinical findings in this study especially in beef group where additional factors like suckling calves increased cessation of ovarian activity and prolonged resumption of ovarian activity. Other factors like uterine infection and NEB were also found to be contributory to ovarian cessation after calving.

Although many studies have found that postpartum ovulation is delayed in dairy cows that yield a high amount of milk (Lucy, 2001; Gong *et al*., 2002), there are other studies who have not found this relationship between milk production and reproductive performance (Lopez *et al*., 2005). Moreover, Bertoni *et al*. (2008) concluded that close relationship between fertility and milk yielding is not visible. However, most dairy cows of the present study produce between 5-10 kg daily and it has a minor effect on reproductive performance of these cows.

5.4.3 Hormonal status

Generally, the levels of this hormone increased progressively during postpartum period. Serum IGF-1 concentration of dairy cows was greater than beef cows across week 3-7 of the postpartum period. The reason for this result could be that most of

the dairy cows sampled resumed ovarian activity during week 4 of postpartum compared with the beef cows that suffered from prolonged anestrus status. This result agrees with many studies that have demonstrated a clear correlation between blood IGF-I levels and ovarian function in postpartum cows. Thatcher *et al*. (1996) found that anestrous cases in dairy cows had lower blood IGF-I levels than cows that had begun ovarian cyclicity earlier after calving, and a similar thing has been found for beef cows (Roberts *et al*., 1997).

IGF-I factor plays a vital role in steroidogenesis and mitosis in thecal and granulosa bovine cells (Lucy, 2000). The same previous study concluded that the reduction in the concentration of the IGF-I in the follicular fluid may reduce the response to both FSH and LH hormones and may develop the disorder in ovarian steroidogenesis process. Ovarian follicular cells play a vital role in secretion and action of IGFs factors.

Our findings revealed that concentration of IGF-1 was lower in cows with SCE in both dairy and beef groups when compared with healthy animals and this may be due to the effect of inflammation and toxin production by bacteria on hypothalamus-pituitary axis and this could have an effect on ovarian resumption. These results agree with many previous studies that have reported a significant relationship between uterine health and resumption of ovarian activity after calving (Huzzey *et al*., 2009; Burke *et al*., 2010). The synergistic relationship between IGF-I and gonadotropins are very important for differentiation and the growth of the ovarian follicle (Spicer and Echternkamp, 1995). Follicular steroidogenesis in postpartum cows is related with LH hormone secretion as well as increasing blood IGF-I concentrations (Beam and Butler, 1999). The independent role of each hormone to follicular function is not easy to establish, however, because both serum IGF-1 and LH pulsatility level increase after calving especially when nutrition level is improved, this lead to an early resumption of follicular activity. Unfortunately, the limitation of this present study was that FSH and LH analysis was not in the scope of this study.

Most beef cows of the present study tend to have prolonged anestrus postpartum period. In addition to the effect of suckling calves on ovarian activity, NEB that occurs in early postpartum cows also probably plays a role because nutritional status partially controls the synthesis and secretion of IGF-I. Serum IGF-1 is decreased in cows after calving when the requirements of the energy exceed the level of nutrient intake (Spicer *et al*., 1990; Beam and Butler, 1999). The present study did not show a significant difference in serum IGF-1 in both healthy and SCE beef groups and most of these animals suffered from prolonged anestrus.

The results in the present study indicated that the levels of progesterone were low during the early postpartum period and it was less than 1ng/mL in blood serum until day 50 in postpartum beef group. These results agree with others studies that confirmed the anestrous postpartum case in beef cows after calving due to the many

reasons like suckling of calves, nutritional cause, and environmental reason (Quintans and Vázquez, 2002). The resumption of ovarian activities for cows during postpartum period requires many essential factors such as endocrine and metabolic competence. Progesterone (P_4) is the main marker for luteal activity because the formation of a CL is main sign of the resumption of ovarian activity (Roche *et al.* 2000). The levels of progesterone is usually at its minimum until the formation of the first postpartum CL (Lewis, 1997) and this is associated with an elevated concentration to more than 1.0 ng/mL in serum within days after the ovulation (Stevenson and Britt, 1979).

The level of serum progesterone in our finding in dairy cows increased progressively until it reached more than 1ng/mL at week 5 and 6 after calving. This may be due to growing follicles (9 mm) that developed and further ovulated and subsequently lead to the formation of the CL and thus elevates serum progesterone. McDougall and Compton (2006) confirmed in a previous study that grazing cows that yield less milk daily were more fertile than high yielding cows in confinement. And this agrees with our results because dairy cows in this study depended on grazing system and the milk production was not very high (5-10 kg/ day) compared with high production of milk (mean 30 kg/ day) on commercial dairy farms.

The current study showed increased serum progesterone levels in healthy cows compared with cows having SCE in both beef and dairy cattle. These results agree with many previous studies that have reported that a significant relationship between uterine health and resumption of ovarian activity after calving (Huzzey *et al*., 2009; Burke *et al*., 2010).

The present study reported the increased concentration of estradiol in the healthy group in both dairy and beef animals compared with SCE group. Most of the cows with a high rate of uterine bacterial contamination during the early postpartum period are associated with the reduction of ovarian follicle size and low secretion of estradiol from the preovulatory follicle (Sheldon *et al*., 2002). Our findings in this study also agrees with this as most SCE cows had lower levels of estradiol until week 7 after calving especially in beef cows.. The concentration of estradiol was higher in healthy dairy group and this explains why most of these cows in the present study had follicles with larger diameter (9-12 mm) at week 5 and subsequently resumed ovarian cyclicity.

5.4.4 Body condition score, Non-esterified fatty acid and β-hydroxybutyric acid

Cow's body condition score represents the quantity of subcutaneous body adipose tissue (Ferguson *et al*., 1994) and this is related to the reproductive performance. To get a good scoring, cows should be scored during the dry and early lactation interval to put a suitable plan to avoid negative energy balance during the postpartum period.

The present study showed decreased BCS of the animals in both beef and dairy cows during postpartum period.

Cows with SCE had BCS of ≤ 2.5 ($P < 0.05$) at week 2 and 4 after calving compared to healthy dairy cows (BCS ≥ 2.7). This result agrees with a previous study that reported the occurrence of endometritis cases in cows that were four weeks after calving and usually have lower body condition scores (Heuer *et al*., 1999). Most of the cows that suffered enddometritis at the beginning of the postpartum period due to low energy intake consequently experienced serious NEB (NEFA 1.4 mmol/dL; BHAB 3.7 mmol/dL) after calving and this led to decrease the whole number of neutrophils and lymphocytes compared with cows that suffered from mild NEB (NEFA 0.3 mmol/dL; BHAB 0.5 mmol/dL) (Wathes *et al*., 2009). These researchers also found that in cows that are two weeks after calving most had severe NEB and there was lack of proper inflammatory responses, while the inflammatory process was different and better in the endometrium of cows that had mild NEB (Wathes *et al*., 2009). Hammon *et al*. (2006) have also demonstrated in a previous study that cows that had serious NEB during the peripartum period had impaired function and activity of blood neutrophils.

This study has shown that beef cows with SCE had low BCS after calving and during the ensuing postpartum period as compared with healthy cows. There are several factors that affect the health of postpartum beef cows and these includes postpartum disorders, NEB, suckling calves and poor management. All these factors influence BCS after calving and consequently suffer from SCE, postpartum anestrus, and impairment of the reproductive performance in future.

The cows of the present study tend to lose body weight and body condition scores after calving and at third week -calving. One study reported that most of the cows that have low BCS early after calving have risk factor on their reproductive performance by increasing the interval from calving to conception and pregnancy loss (Santos *et al*., 2009a). This agrees with our results as about 38 % and 80 % of dairy and beef cows respectively had a cessation of ovarian activity during the postpartum period. According to a recent study by Ribeiro *et al*. (2013), the optimal BCS for grazing cows was supposed to be between 3-3.25 after parturition to minimize clinical and subclinical postpartum disorders. Thus, all management programs during peri prepartum period should be tailored towards maintaining a body condition score within 3.00 to 3.25 to avoid the occurrence of metabolic and uterine diseases and consequently reduce reproductive performance of these cows (Ribeiro *et al*., 2013). However, this disagrees with our findings, as such, the cows that had a body condition score of 3 at the time of calving (most of them being beef cows) had a prolonged resumption of ovarian activity during the postpartum period a likely reason for this is the prolonged postpartum anestrus due to the effect suckling by calves. Other reasons may be due to differences in breed, management system or breed differences. Again beef cows in this study were exposed to the reduced intake of dry material after calving which could lead to lowered BCS. Also, the current

study showed that most of the cows especially in dairy cows with BCS of more than 2.5 showed ovarian cyclicity better than these cows with lesser BCS which further tend to suffer from NEB and prolonged postpartum anestrus. This result agrees with a previous study where it was reported that a significant relationship exist between the status of nutrition in cow farms and reproduction in cows located in tropical areas (Montiel and Ahuja, 2005). The imbalance between nutrition intake and the production demands of these cows develops postpartum anestrus especially when these cows depended on grazing to meet their feed requirements (Jolly *et al*., 1995). The cows in both dairy and beef in the present study almost depended on grazing system to meet their demands.

The NEB is considered as one of the most common reasons that have an adverse effect on the ovarian resumption after calving in dairy and suckled beef cows. NEB disrupts immune function which may further predispose cows to uterine infection and subsequently reduce their ability to overcome postpartum uterine infection (LeBlanc 2010). Interestingly, a greater degree of negative energy balance is usually represented usually by increased non-esterified fatty acids (NEFA) and beta-hydroxybutyrate (BHBA). The level of blood NEFA factor reflects the magnitude of fat mobilization, while the level of blood BHBA reflects the oxidation of fat in the liver (LeBlanc, 2010).

In the present study, Serum NEFA and BHBA level in both SCE dairy and beef groups had a higher concentration compared with healthy ones. These results correspond with a previous study that reported cows that suffered from SCE and metritis had decreased energy intake and developed increased concentrations of serum NEFA and BHBA until week 4 after calving (Hammon *et al*., 2006). Another study by Galvao *et al*. (2010a) revealed that cows that had a uterine infection like metritis and subclinical endometritis develop an increasing level of NEFA and BHBA than healthy cows.

The present findings agree also with a study by Wathes *et al*. (2009) who showed that most of the cows that suffered from poor energy intake at the beginning of the postpartum period consequently faced serious NEB after calving, had decreased whole number of neutrophils and lymphocytes compared with cows that suffered from mild NEB.

Results from the present study disagree with a recent research that demonstrated a high blood glucose concentration accompanied with clinical endometritis, and hazard factor for metritis, and endometritis. Neither serum BHBA nor NEFA concentrations did differ in both endometritis and metritis cows compared with the healthy cows (Bicalho *et al*., 2017). And this may because this study was conducted between 50 days before calving until two weeks postpartum; an increase of glucose during three days after birth was explained by the action of cortisol hormone which secreted around calving period and causes consequently decreased of NEFA and BHBA after two weeks of calving while our findings were conducted between week

3 and 6 postpartum. Moreover, Burke *et al*. (2010) and Dubuc *et al*. (2012) in previous studies did not demonstrate any relationship between the concentration of glucose and NEFA (EB) and endometritis. However, this study has reported decreased in albumin level as an indicator of liver dysfunction due to endometritis. Maybe because the BCS and body weight of SCE cows in these previous studies did not differ than healthy cows and this did not influence energy balance of these cows. Our findings showed a significant decreased in BCS in SCE compared with healthy cows in both dairy and beef animals.

In the current study, the acyclic cows of the dairy group had a lower BCS (significant difference at week 2 and 4) and greater levels of NEFA and BHBA than cyclic cows during the postpartum period. This agrees with many studies that confirmed the relationship between severe NEB and delayed first ovulation after calving (Wiltbank *et al*., 2006; Santos et al., 2009a; Burke *et al*., 2010; Giuliodori *et al*., 2011; Castro *et al.*, 2012). Cows that have a high concentration of blood NEFA had increased occurrence of anovulation. Despite the significant relationship between blood level of NEFA, insulin and the duration of the anovulatory period after calving previously (Butler, 2003; Wiltbank *et al*., 2006), other studies did not find any difference in the serum level of insulin and NEFA between healthy, endometritis, ovulatory and anovulatory dairy cows (Krause *et al*., 2014). And this may be explained because the anovulatory case in dairy cows may be associated more with the inflammatory status than the stress of nutritional conditions that are represented by the degree of the lipid mobilization and the production of milk (Butler, 2003). This explanation, however, does not fully support our findings due to the presence of a high number of healthy beef cows that suffered from prolonged ovarian cessation condition despite these cows were clear from uterine diseases. The dairy cows with high milk yield are more exposed to the adverse impacts of NEB: however, there is a debate about the effect of high milk production with ovarian activity and a delayed first postpartum ovulation.

Most dairy cows in the current study had low milk production (average 5-10 kg/day) in both the healthy and SCE groups and the reason for the increased NEFA concentration may be because the energy balance that plays a vital role in grazing cows, due to the low dry material intake and maybe this develops decreasing of caloric energy diets that consumed by these cows (Bargo *et al*., 2003).

5.5 Conclusion

The current study showed the lower occurrence of SCE in both dairy and beef groups and especially in beef cows, and heifers were more prone to suffer from SEC than multiparous cows in week 4 postpartum. Most beef cows of the study showed prolonged postpartum anestrus and were mainly associated with ovarian cessation and consequently leading to increased days open and this related to many causes like uterine infection, suckling and lower BCS after calving. The ovarian resumption in dairy cows group was faster in healthy cows compared with cows that had SCE, and

BCS lower than 2.5 during the early postpartum period. The study also revealed increased levels of NEFA and BHBA in SCE animals compared with healthy cows during most of the weeks of the study in both dairy and beef groups.

CHAPTER 6

DETERMINATION OF SERUM ACUTE PHASE PROTEINS AND INFLAMMATORY CYTOKINES IN HEALTHY AND ENDOMETRITIC POSTPARTUM CATTLE

6.1 Introduction

Severe economic losses occur in cows when the uterus is exposed to several types of microbial infection during and after parturition (Sheldon *et al*., 2006). Many types of bacteria play different role in the complex etiology of subclinical endometritis (SCE) (Bicalho *et al*., 2010). Although the uterus is exposed to different types of microorganisms after calving, more than 70 % of postpartum cows can clear uterine contamination through immune mediated responses, but 17 to 37 % of these postpartum cows may develop clinical endometritis (CE), whereas 14 to 53 % of postpartum cows are exposed to SCE (Gilbert *et al*. 2005; Cheong *et al*. 2011; Madoz *et al*. 2014)

Cellular and humoral immunity play an important role in the clearance of uterine infectious agents. Inflammation of the endometrium develops complicated signaling processes including the early detection of microorganism components by innate immune cells by Toll-like receptors, the secretion of the proinflammatory cytokines factors such as tumor necrosis factor-α (TNF-α) and different interleukins (IL)). Moreover, the process involves the mobilization of white blood cells, mainly neutrophils, into the endometrium and consequently phagocytosis of pathogenic microorganismsms (Sheldon *et al*., 2009).

The proinflammatory cytokines factors like TNF-α, IL-1β, and IL-6 and chemokines (IL-8) play an essential role to stimulate leukocytes like neutrophils diapedesis and acts as a chemoattractants and to promote white blood cells for phagocytosis (Singh *et al*., 2008). These inflammatory cytokines also help and stimulate in the secretion of different acute phase proteins (APPs), like haptoglobin (Hp), acid glycoprotein and serum amyloid A (SAA) (Tothova *et al*., 2008). The function of these APPs is to clear uterine contaminants through adjustment of other immune proteins and stimulation of phagocytosis. APPs also play other roles by providing protective function against any damaging impact of different enzymes secreted during the inflammation process.

APPs are secreted mainly by the liver, and their levels in the blood of cows increase during the first weeks after calving, in response to uterine diseases (Tothova *et al*., 2008). Although APPs are synthesized in other organs outside the liver, the possibility of the endometrium, to syntesize and secrete APPs is still not confirmed (Davies *et al*. 2008). However, another previous study by Chapwanya *et al*. (2013)

had concluded the possibility of SAA secretion by uterine endometrium cells in cows.

APPs have the significant effect in many steps of the inflammation process and thus may serve as good indicators of different types of infection in cows (Tothova *et al*., 2012). Haptoglobin (Hp) and serum amyloid A (SAA) are considered as essential positive biomarkers for exposure to pathogens (Tothova *et al*., 2014). Serum amyloid A is a lipoprotein substance secreted within 24–48 h after infection, as a protein of the first line of response and SAA production is dependent mainly on proinflammatory cytokines IL-1 and TNF-α (Tothova *et al*., 2014). Hp is considered as a protein of the second line of inflammatory response, whose production is controlled by the proinflammatory cytokine IL-6 and its high concentration may be a characteristic of long and or subacute inflammation (Tothova *et al*., 2014). Brodzki *et al*. (2015a) found a significant increase in the high concentrations of serum SAA in cows that suffer from endometritis as compared to healthy cows. Furthermore, the concentration of Hp was significantly greater in uterine washings and serum of cows that suffered from SCE. Such alteration in the level of APPs may indicate the existence of chronic inflammation of the uterus. The continued presence of an elevated concentration of Hp factor in the uterine washings and serum in cows suffered from SCE is very important from a clinical point of view. Hp is considered as a good indicator of inflammatory response of the uterus in cows. Most results of similar studies found the importance of proinflammatory cytokines and Hp in serum and uterus as an important diagnostic indicator of cows that had SCE (Brodzki *et al*., 2015a and 2015b).

Most of the previous studies on this subject was conducted on dairy postpartum cows and there is paucity of information in beef cows. The objective of this study was to determination and compare the concentrations of IL-6, IL-8, Hp and SAA between healthy postpartum and SCE groups in both beef and dairy postpartum cattle.

6.2 Materials and methods

6.2.1 Animals

The same animals that used in the previous chapter 5 (5.2.1).

6.2.2 Endometrial cytological

Endometrial cytological samples were collected from the cows by using the modified CB as described in the chapter 3.

Endometrial threshold value ≥ 8% was used (Madoz *et al*., 2013; Ricci *et al*., 2015) to determine the SCE occurrence in the farms between 20 and 30 days postpartum.

6.2.3 Blood samples

Blood samples (10 ml) were collected once weekly from week four until the week seven after calving. The same procedure was done as mentioned in chapter 5 (5.2.5).

6.2.4 Measurement of interleukins and acute phase proteins in blood serum

The concentrations of IL-6, IL-8, Haptoglobin and SAA measured by using an enzymatic method. The QAYEE® kit (Qayee-bio for life science, China) was used for IL-6, IL-8, Haptoglobin and SAA serum levels. Sensitivity was 7.8 pg /mL 31.2 pg /mL, for IL-6, IL-8 and 7.8 ng /mL, and 12 μg/mL for haptoglobin and SAA, respectively. The Intra- and interassay coefficients of variation were 4.8% and 10.7% for IL-6, and 5.6 % and 11.4% for IL-8, and 3.8% and 12.2% for Hp, and 5.4% and 13.4% for SAA.

6.2.5 Statistical analysis

All the statistical methods were performed by SPSS software (version 18.0, IBM SPSS Inc., Chicago: USA). All values are expressed as the mean ±standard error of the mean. The Shapiro–Wilk test was used to confirm the normal distribution of the traits examined. The results of the control and experimental groups were compared using the Student t-test to determine statistical significance. Statistically significant differences for the samples collected on w 4, w 5, w 6 and w 7 postpartum in the two groups were calculated using one-way ANOVA as well as Tukey and Duncan post hoc tests at the probability threshold $P < 0.05$.

6.3 Results

The study showed that there was no significant ($P > 0.05$) difference in serum IL-6 levels between healthy dairy group and cows having SCE at week four postpartum. However, it was significantly greater in SCE group on subsequent weeks 5, 6 and 7 among dairy cows. The IL-6 level in healthy dairy group was lower at week 6 and 7 ($P < 0.05$) than week 4 and five postpartum while there was no significant difference in serum IL-6 levels during 5-7 week postpartum in dairy cows having endometritis (Fig. 6.1). In the beef group, there was a significant difference ($P < 0.05$) in IL-6 level between healthy cows and cows with SCE at week 4, 5 and 7 postpartum and within cows with SCE, week 4 and 5 was higher ($P < 0.05$) than week 6 and 7 postpartum. In healthy beef cows, IL-6 concentration was lower with a significant difference than any other postpartum weeks (Fig. 6.2).

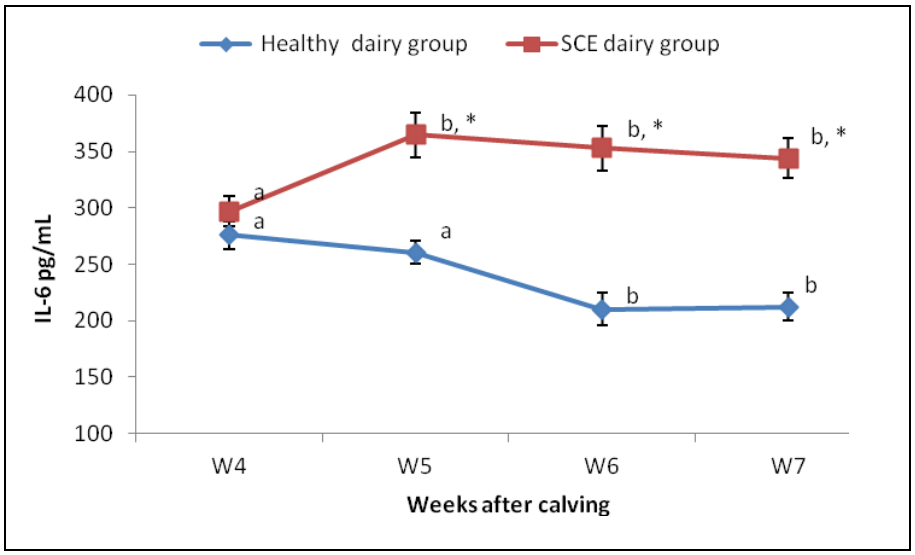

Figure 6.1 : Serum IL-6 levels Mean ±SEM in both healthy (n=8) and SCE (n=8) dairy groups.
* indicate significant difference at p< 0.05 between healthy and SCE groups.
[ab] Means values among weeks in same group with different superscripts indicate significant difference at P < 0.05.

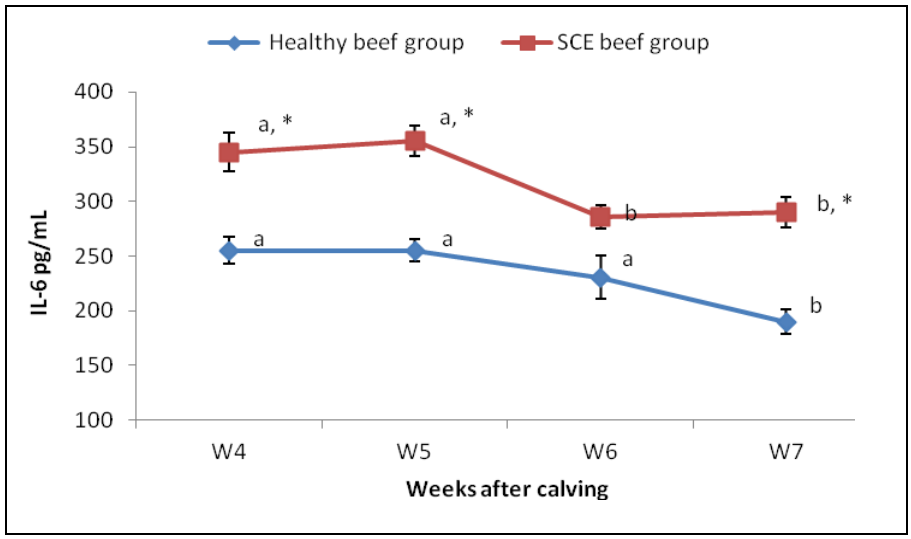

Figure 6.2 : The serum IL-6 levels Mean ±SEM in both healthy (n=12) and SCE (n=12) beef groups.
* indicate significant difference at p < 0.05 between healthy and SCE groups.
[ab] Means values among weeks in same group with different superscripts indicate significant difference at P < 0.05.

The level of IL-8 in both SCE dairy and beef groups was greater (P < 0.05) at week 5-7 than in healthy groups. The concentration of IL-8 had gradually decreased in cows with SCE in dairy and beef animals with the advance of the postpartum period.

Week 6 and 7 postpartum cows had a lower level ($P < 0.05$) at dairy healthy group than weeks 4 and 5 (Fig.6.3). At week 7, there was a significantly lower concentration ($P < 0.05$) of IL-8 among healthy beef cows than at any other week postpartum (Fig. 6.4).

The study revealed that dairy SCE cows had greater concentrations of Hp compared with healthy cows, which were significant ($P < 0.05$) at week 5-7 postpartum, but there was no significant difference along 4-7 postpartum SCE dairy cows. There was a decrease in serum Hp level with a significant difference at week 6 and 7 in healthy dairy cows when compared to week 4 and week 5 postpartum (Fig.6.5).

Beef cows also showed a significant increase in serum Hp levels at week 5-7 in cows with endometritis cows than healthy cows. it was also higher at week 7 than during week 4to 6 in beef cows with SCE. The concentration of Hp at week 4 showed a significant increase in the healthy beef cows as compared to subsequent weeks (Fig. 6.6).

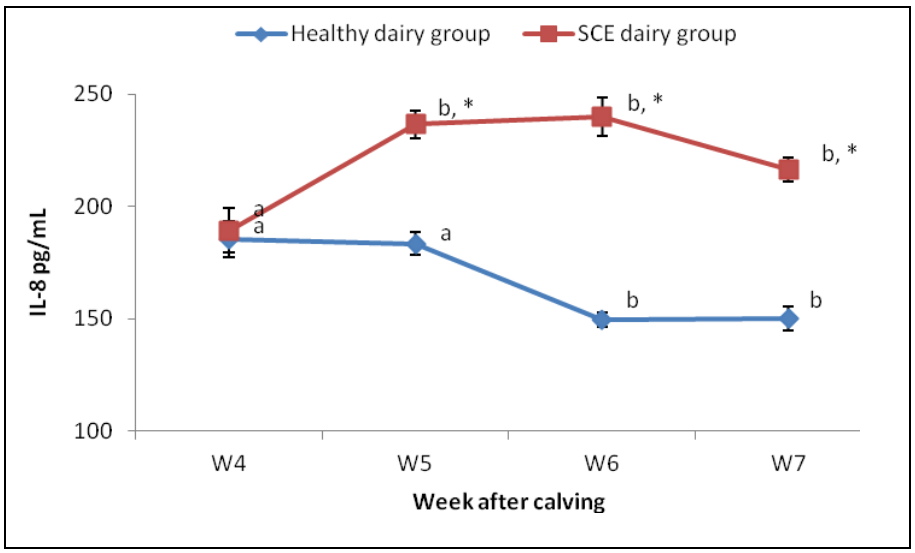

Figure 6.3 : Serum IL-8 levels Mean ±SEM in both healthy (n=8) and SCE (n=8) dairy groups.
* indicate significant difference at $p< 0.05$ between healthy and SCE groups.
[ab] Means values among weeks in same group with different superscripts indicate significant difference at $P < 0.05$.

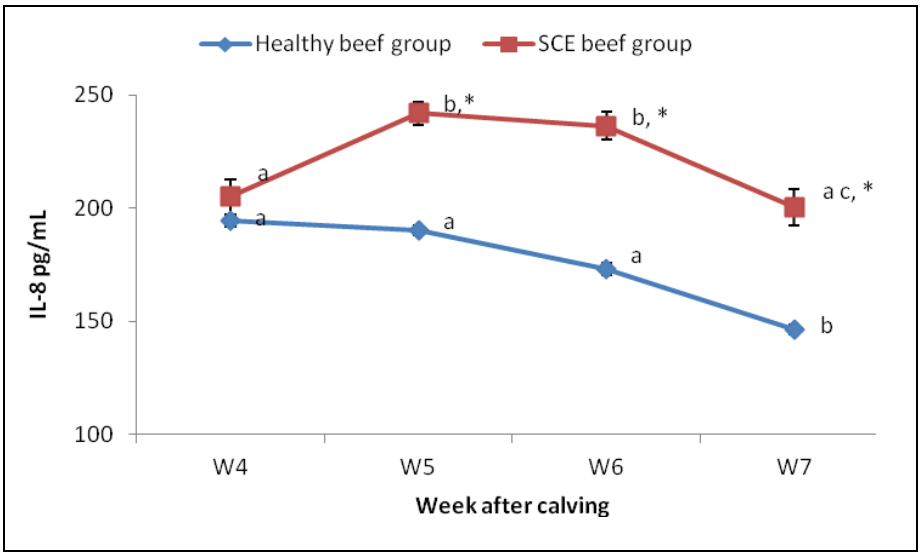

Figure 6.4 : Serum IL-8 levels Mean ±SEM in both healthy (n=12) and SCE (n=12) beef groups.
* indicate significant difference at p< 0.05 between healthy and SCE groups.
[ab] Means values among weeks in same group with different superscripts indicate significant difference at P < 0.05.

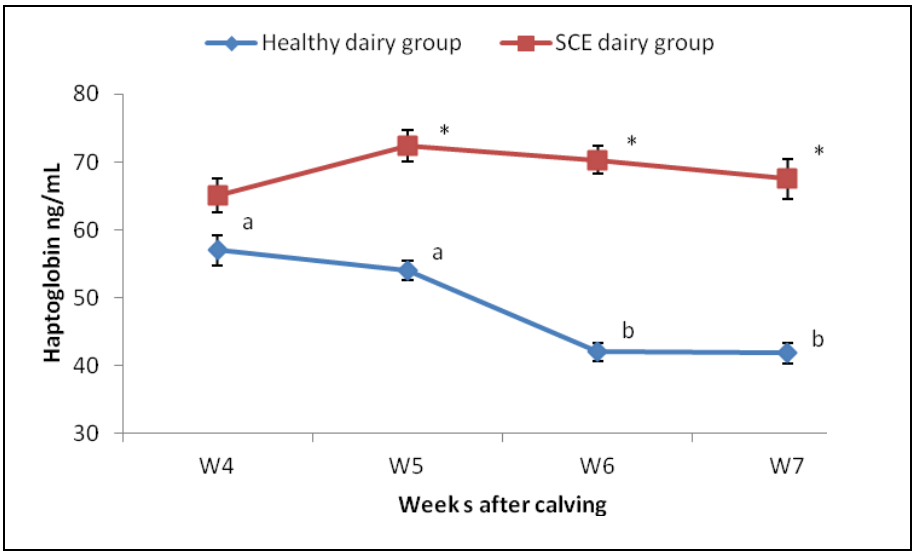

Figure 6.5 : Serum Hp levels Mean ±SEM in both healthy (n=8) and SCE (n=8) dairy groups.
* indicate significant difference at p< 0.05 between healthy and SCE groups.
[ab] Means values among weeks in same group with different superscripts indicate significant difference at P < 0.05.

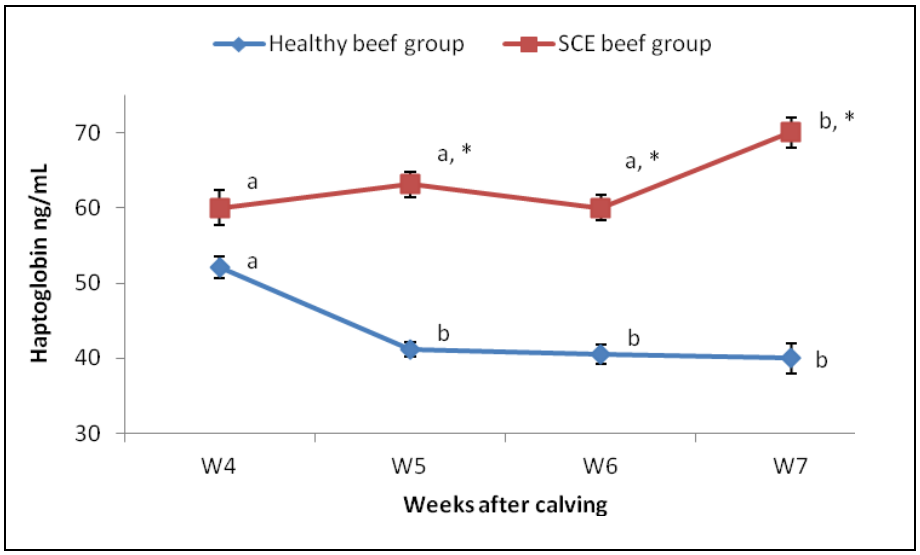

Figure 6.6 : Serum Hp levels Mean ±SEM in both healthy (n=12) and SCE (n=12) beef groups.
* indicate significant difference at p< 0.05 between healthy and SCE groups.
[ab] Means values among weeks in same group with different superscripts indicate significant difference at P < 0.05.

The study showed an increasing serum level of SAA at week4 to 6 in SCE dairy group compared to healthy ones. SAA level was decreased significantly with advance of postpartum period in both SCE and healthy groups (Fig. 6.7). In beef group the level of SAA was (P < 0.05) higher at week 5 and 6 in SCE group than healthy group and also level of SAA showed a decreasing trend in subsequent weeks of the study (Fig. 6.8).

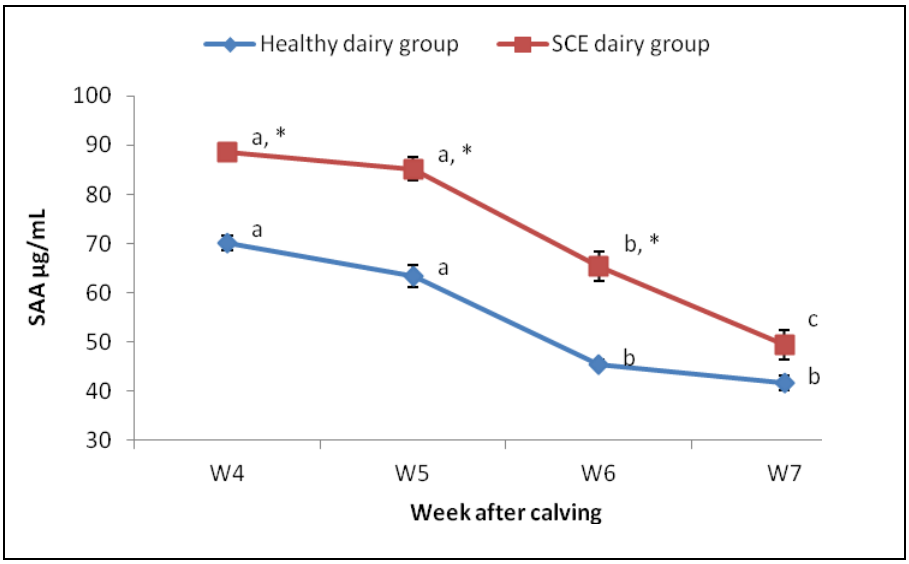

Figure 6.7 : Serum SAA levels Mean ±SEM, in both healthy (n=8) and SCE (n=8) dairy groups.

* indicate significant difference at $p < 0.05$ between healthy and SCE groups.

[abc] Means values among weeks in same group with different superscripts indicate significant difference at $P < 0.05$.

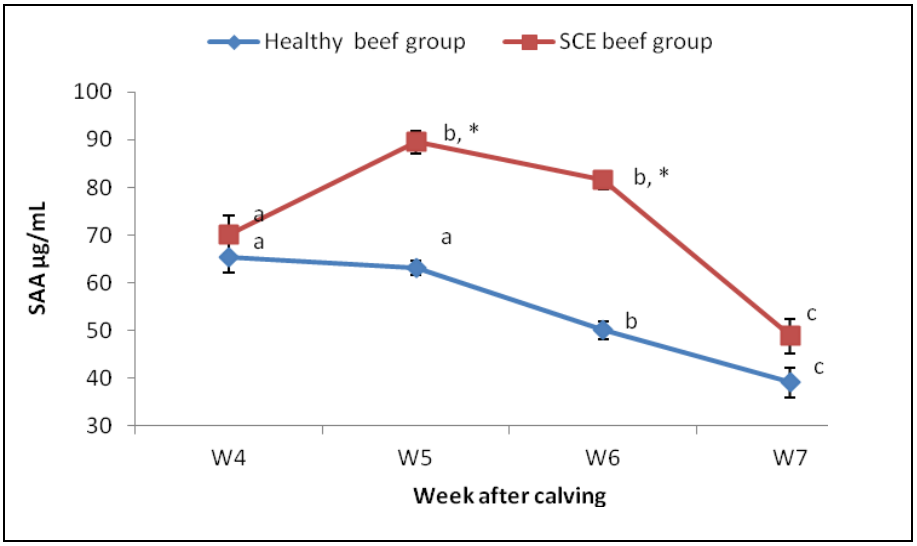

Figure 6.8 : Serum SAA levels Mean ±SEM in both healthy (n=12) and SCE (n=12) beef groups.

* indicate significant difference at $p < 0.05$ between healthy and SCE groups.

[abc]Means values among weeks in same group with different superscripts indicate significant difference at $P < 0.05$.

6.4 Discussion

The aim of this part of the study was to compare and evaluate the concentrations of proinflammatory cytokines: IL-6, chemokine IL-8, and APPs (Hp and SAA), in the serum of both beef and dairy cows with and without SCE.

Interleukins are a kind of immune protein factors that are expressed by white blood cells. They are secreted by different body cells and play a vital role in the function and activity of the immune system. The lack of the protective role of these interleukins can cause immune deficiency and autoimmune diseases in cows (Menachem *et al*., 2011; Mossallam *et al*., 2015).

The immune reaction after parturition in animals is associated with elevated levels of many different proinflammatory cytokines and APPs which are subsequently followed by the mobilization of white blood cells (neutrophils) and is essential for the uterine health in animals (Butterfield *et al*., 2006).

The present study assessed the level of proinflammatory cytokines (IL-6, IL-8), and APPs (Hp and SAA) in serum of cows with SCE and healthy during week4 until week seven postpartum period. The current study showed no significant difference in the concentrations of serum IL-6 in SCE and healthy dairy cows at week 4 postpartum. Similarly, a previous study did not find any difference between the concentrations of serum TNF-a, IL-6, and IL- 10 in the first 5 days of postpartum period in healthy cows and cows that were later diagnosed with SCE (Brodzki *et al*., 2015a). This result confirms that the inflammatory response in uterus occurred in some cows despite the initial status was similar (Brodzki *et al*., 2015a).

Our study showed increased levels of serum IL-6 and IL-8 during weeks 5-7 postpartum in cows with SCE compared with healthy cows in both type of cattle. This result may be due to an increased immune response, especially in the uterus, and as a result, levels of IL6 and IL-8 in the serum of cows with SCE becomes increased. It is noteworthy that IL-6 is an essential pro-inflammatory cytokine, and plays a vital role in many aspects of the inflammation process like the induction of fever in animals, elevation of vascular permeability and stimulation APPs secretion by the liver (Van Snick, 1990).

One of the most common causes for the increased level of IL-6 is the continuous production by uterine endometrium cells, as a result of the activity of endometrium Toll-like receptors and the recognition of the pathogen that invade the endometrium in postpartum period (Sheldon *et al*., 2009; Turner *et al*., 2012). This reaction is considered the key activation factor to the uterine immune response, which leads to the attraction of leucocyte and enhancement of the activity of neutrophils and macrophages for phagocytosis. Both uterus and peripheral leukocytes could be activated by microorganisms (mainly phagocytes), and could secrete

proinflammatory cytokines, contributing to increasing their levels in the uterine lumen (Singh *et al*., 2008).

Many previous studies reported infiltration of neutrophils to the endometrium of cows that suffered from SCE and this was due to exposure the uterus of these cows to many different pathogens microorganisms after calving (Kasimanickam *et al*., 2004; Sheldon *et al*., 2006; Brodzki *et al*., 2015a; 2015b).

The concentrations of IL-6 and IL-8 in healthy cows started to decrease gradually from week 4 until week 7 in both dairy and beef groups in the current study and this can be explained due to the absence of inflammatory reaction in these cows compared with cows had SCE and were characterized by high levels of proinflammatory agents.

Proinflammatory cytokine-like (IL-8), is also as considered as one of the major neutrophil chemokine, which was primitively known as a chemotactic agent produced by activated white blood cells that promote the migration of leukocytes like neutrophils and lymphocytes, have an essential function for inflammatory response and acute and chronic diseases (Tseng and Leibert, 2009). The concentrations of IL-8 in week 4 postpartum were low in cows with SCE compared with same cows in following weeks postpartum. This finding could have been related to induce stimulation by pathogenic microorganisms that begun after week 4 when the inflammation of endometrium of SCE cows have occurred. After calving and at the early weeks of the postpartum period, healthy cows get rid of most of microorganism contaminating the uterus compared with the cows that later suffered from endometritis. Sheldon *et al*. (2009) confirmed in a previous study that most of the bacterial contamination of the uterus after parturition is cleaned within three weeks after calving, and such cows are sterile during 6–8 weeks postpartum. The local immune response is not hight as because the level of uterine bacterial contamination is not high or virtually absent after birth. The concentrations of cytokines agents and APPs are also low, which was found in healthy cows while the cows that suffered SCE have a huge local uterine immune reaction and more increased proinflammatory mediators.

Our results agree with previous studies that confirm higher levels of proinflammatory cytokines (TNFa, interleukin-1b, IL-6, and IL-8) in serum of dairy cows with SCE compared with healthy cows (Brodzki *et al*., 2015a). Moreover, another study concluded that postpartum dairy cows with SCE expressed greater concentration of TNF-a, IL-6, and IL-8 mRNA than healthy dairy cows (Ghasemi *et al*., 2012). A previous study by Galvão *et al*. (2011) also reported, increasing pro-inflammatory cytokines (IL-1_, IL-6, and IL-8) in SCE cows only during weeks 5 and or 7 postpartum compared to control (healthy) cows. However, the present study used concentration of interleukins in serum of postpartum cows while the two previous studies depended on the express gene (Real-time RT-PCR) of these cytokines as a marker to compare between healthy and SCE dairy cows.

Hoffmann *et al*. (2002) concluded in their study that infection caused by viral and bacterial agents may induce rapid immune response and high level of IL8 up to 100-fold. Also another study found a significant increase of chemokine IL-8 gene expression in SCE cows compared with healthy cows (Galvao *et al*., 2011). This study conlcuded that a minimum local level of inflammatory cytokines in the uterus after calving may impair the inflammation and clearance of uterine contaminants and thus lead to infection and endometritis in these cows. A previous study by Ghasemi *et al*. (2012) also highlighted the importance of IL-8 to predict endometrial infection. Similarity, most cows in the current study that had SCE and so were suffering from bacterial contamination (Mostly *E. coli* 42.8%) after calving (Chapter 4, Table 4.1) and consequently uterine infection and this lead to an induced immune response by increased IL-6 and IL-8 in uterine and serum of these cows.

A study by Mossallam *et a*l. (2015) that involved the investigation of IL8-expression in the uterus of healthy and endometritis in Egyptian buffalo cows reported a great increase in IL8 expression (26.6-fold) in endometritis compared with healthy buffalo. Moreover, the study showed that concentration of IL-6 was higher in most of the weeks of the study period and this indicates that these cytokines might be strongly related to the development of uterine inflammation in postpartum dairy cows (Brodzki *et al*., 2015a).

Despite the foregoing, our study has depended serum assessment to determine proinflammatory and did not use DNA and gene expression, most of the cows that had SCE showed increased the levels of IL-6 and IL-8 during most of the weeks of the study compared with healthy cows.

The current study also showed decreased concentrations of IL-6 and IL-8 in advance of the postpartum period in most cows with SCE and this finding may be due to the ability of the most of the cows with time to get rid of the pathogenic infection in the following weeks after uterine infection. In contrast, another study revealed that no significant difference exists in the levels of IL-6 and IL-8 in serum of healthy and cytological endometritis cows. However, differences in cytokines concentration were noticed in the uterine flush of endometritis cows (Kim *et al*., 2014). This finding may be explained due to the minor intensity of uterine infection in the previous study that causes local inflammatory reflex without increasing of serum cytokines. Also, one a study by Ishikawa *et al*. (2004) did not find a significant concentration of IL-6 difference in both the serum of endometritis and healthy cows. A previous study on the expression of IL-1b, IL-8, and TNF-a mRNA using real-time reverse transcription– polymerase chain reaction experiments showed significantly higher values in cows with clinical and subclinical endometritis than healthy cows between 21 and 27 days postpartum. In contrast, IL-6 mRNA expression was not influenced by uterine inflammation (Fischer *et al*., 2010). The difference between results that have obtained by different studies may be due to the different methods that used, times of sampling and breed differences. Also, our study involved both dairy and beef cows and due to the lack of studies about these interleukins in beef postpartum

cows, this study depended on dairy cows as a guide to explain most of results findings.

The APPs are categorized as inflammatory proteins factors that are elevated in their concentration by 25% or more during inflammatory response (Whicher *et al*., 1991).

The haptoglobin (Hp), serum amyloid A (SAA) are the most frequently demonstrated protein factors in cattle during inflammation (Dowling *et al*., 2002). APPs have been reported to have significant effect in many steps of the inflammation process and thus may serve as good indicators of different types of infection in cows (Tothova *et al*., 2012). The haptoglobin (Hp) and serum amyloid A (SAA) are considered as essential positive biomarkers (Petersen *et al*., 2004; Tothova *et al*., 2014).

Serum Hp level decreased to baseline values in healthy cows by the third week after calving, while the level of serum SAA reached baseline value in the first-week postpartum period, and this finding is associated with completion the involution of the uterus by 26–52 days after calving (Chan *et al*., 2010).

Our results indicated increased serum SAA and Hp level during most weeks of the postpartum period for both SCE dairy and beef groups compared with healthy groups and this agrees with other studies that have confirmed that APPs, especially Hp, and can be used as a marker to diagnosis uterine inflammatory reaction in dairy cows (Ceciliani *et al*., 2012). The use of Hp as an indicator for diagnosis of SCE is very useful as there were a few previous studies that included this matter (Brodzki *et al*., 2015a; 2015b).

The level of Hp was higher during 4-7 weeks postpartum in the serum of cows with SCE. These concentrations of APPs may suggest the persistence of chronic inflammation of the uterus from the early postpartum period to day 50 postpartum.

In contrast, Yasui *et al*. (2014), reported that a weak relationship between serum Hp and cytological endometritis, or with decreasing reproductive performance in postpartum cows between 40 and 60 days postpartum. The same study reported that NEB during the early postpartum period is associated with SCE and not necessarily is occurred with systemic inflammation reaction. The type of infection and intensity of microbial contamination after calving may affect the immune response, as well as the difference in the breed, age, and environment on the size and type of the immune response in these cows between different studies.

Our results confirmed the results of the most of previous studies about a significant relationship between IL-6 and the level of Hp during inflammation process, most the weeks of the study the both concentration of Hp and IL-6 was high in infected cows

cpmpared with healthy cows (Tothova *et al*., 2014). Hp, in turn, it is a protein factor of the second line of reaction, whose production is regulated by the proinflammatory cytokines (IL-6), and its increased concentration is characteristic of long and less severe inflammatory processes (Petersen *et al*., 2004; Tothova *et al*., 2014). Hp concentration was higher most the duration of the present study coinciding with the increasing of the IL-6, this confirms the strong relationship between them in SCE cows, also because increasing Hp is often associated with chronic infection in these cows.

Many previous studies concluded increasing Hp level at postpartum uterine infection in cows (Huzzey *et al*., 2009; Burke *et al*., 2010; Schneider *et al*., 2013) and this agrees with our findings in both beef and dairy SCE groups. The levels of Hp were increased from week 4 and had a significant difference during the following weeks (week 5-7). Also SAA levels were greater in SCE cows compared with healthy ones along postpartum week and this agrees with other previous studies (Brodzki *et al*., 2015a; 2015 b). Serum SAA in our findings was gradually decreased with the advance of the postpartum period in both healthy and SCE beef and dairy cows because it is related with initiated immune response (first line immune) and begin to decrease gradually.

The continuous high level of Hp in the serum and uterine washings of cows with SCE is essential from the clinical point of view because this can serve as a marker to predict inflammation of the uterus in these cows (Brodzki *et al*., 2015a).

Although the level of both Hp and SAA in SCE group was significantly greater than healthy cows, the studies confirmed that Hp is a better indicator of postpartum uterine disease than SAA (Chan *et al*., 2010). This result disagrees with another study which concluded that serum Hp increased in dairy cows with acute metritis, but not in chronic endometritis (Skinner *et al*., 1991). Our finding found IL-6, IL-8, and Hp was better markers for postpartum cows that suffer from SEC compared with healthy cows because these factors were increased most weeks of our study.

Increasing Hp level during two months after birth may affect the reproductive performance by increasing open days of these cows. On the other hand, SAA concentration was not elevated at all times and it may be associated with acute uterine disease and stress factor in postpartum cows (Chan *et al*., 2010).

Besides inflammation, it must be noted that Hp and SAA concentrations are increased in sera of cows subjected to physical stress, such as transportation, exhaustion, starvation, or parturition, during which serum glucocorticoid concentrations increased. All cows of the present study were already checked to ensure these cows are free signs of inflammation and clinical disease to avoid possible errors in concentration of these proinflammatory and acute phase proteins.

6.5 Conclusion

The current study is considered the first report on the level of cytokines and APPs in the serum of cows that developed subclinical endometritis in the early postpartum period in beef and dairy beef cows in Malaysia.

The current study shows increased levels of both proinflammatory and APPs in both dairy and beef groups with subclinical endometritis compared with healthy cows during 4-7 weeks postpartum. It can clearly be seen that high, IL-6, IL-8, Hp and SAA concentrations in the postpartum period are negatively associated with cow's health status. Our results indicated that the evaluation of the levels of IL-6, IL-8 and Hp in serum can be important diagnostic indicators of SCE in dairy and beef cows as long as these cows are without clinical diseases and not exposed to stress factors

CHAPTER 7

AGREEMENT BETWEEN ENDOMETRIAL CYTOLOGY AND ULTRASOUND EXAMINATION FOR DIAGNOSIS OF ENDOMETRITIS IN POSTPARTUM BEEF COWS

7.1 Introduction

subclinical endometritis (SCE) is the inflammation of the uterine endometrium without mucopurulent material accumulation in the vagina and any systemic symptom (Sheldon *et al*., 2006). SCE is also known as cytological endometritis (Gilbert *et al*., 2005; Dubuc *et al*., 2010). Dubuc *et al*. (2010) described cytological endometritis as "an elevated ratio of polymorphonuclear cells (PMN) in endometrial cytology samples obtained through cytobrush (CB) or low-volume uterine lavage (LVF)."

Precise diagnosis of endometrial infections in cows is hindered by the lack of consensus on an acceptable definition of bovine endometritis (Gilbert *et al*., 2005; Sheldon *et al*., 2006). Most cows experience some degree of endometritis during normal uterine involution after birth. Transrectal palpation of the uterus is the most common method of diagnosing postpartum uterine diseases; however, this method lacks the accuracy to identify endometritis and subsequent reduced fertility (LeBlanc *et al*., 2002; Runciman *et al*., 2008). Several approaches, such as the collection of endometrial and inflammatory cells using a guarded cotton swab (Studer and Morrow, 1978), uterine biopsy (Bourke *et al*., 1997), LVF (Gilbert *et al*., 2005), or CB (Kasimanickam *et al*., 2004), are used to detect cytological endometritis. Mateus *et al*. (2002) found that uterine measurement using ultrasound is convenient and allows for reliable result comparison.

Ultrasonographic intrauterine fluid determination 3 weeks postpartum exhibited good sensitivity and specificity and is reliable for diagnosing endometritis (Kasimanickam *et al*., 2004; Barlund *et al*., 2008).

Most of the previous studies however were performed about endometritis in dairy cows, and limited information is available on beef cows by using ultrasound technique in Malaysia. Thus, this study aimed to evaluate the ultrasound method and its agreement with the endometrial cytology method used to diagnose endometritis in beef cows.

7.2 Materials and methods

7.2.1 Animals

The study was conducted on cows during weeks 4 and 5 postpartum. A total of 53 clinically healthy beef cows (28 Brangus and 25 Kedah–Kelantan breeds) were used. The cows were in the age range of 3-7 years and their body weights were from 300 to 450 kg. Moreover, the cows were managed under a free grazing system and supplemented consistently with feeds consisting of alfalfa, corn silage, beet pulp, cottonseed, soya bean, corn, and barley.

7.2.2 Ultrasound scanning

Ultrasound examination was conducted to determine the uterine cervix diameter and fluid accumulation in the uterine lumen according to procedure described earlier (Kasimanickam *et al*., 2004; Meira *et al.*, 2012). All cows were scanned at weeks 4 and 5 postpartum using B-mode ultrasound attached with a linear probe of 5MHz frequency (Sonosite VET 180 Plus, Bothell, WA, USA). The cows were grouped into two categories: endometritis and healthy cows. Cows with uterine cervix diameter measurement (CD) higher ≥ 5 cm and uterine horns containing fluid in the uterus (FIU), regardless of the amount or nature (hyperechogenic or hypoechogenic), upon ultrasonography, were classified under the endometritis group, as described by Meira *et al*. (2012). A cow was categorized as healthy when its uterine cervix diameter was <5 cm, with no abnormal discharge externally or in the uterus based on ultrasonographic findings, as described by Meira *et al*. (2012).

7.2.3 Endometrial cytology

Endometrial cytological samples were collected at both week 4 and 5 postpartum using a sterile CB Plus GT (Fig.7.1) (Medscand Medical, Germany) modified for bovine use, as described by Madoz *et al*. (2013) in previous chapters. Endometrial threshold values ≥8% were used, as described by Madoz *et al.* (2013), and Ricci *et al*. (2015) to determine the occurrence of endometritis in the farms 20-35 days postpartum.

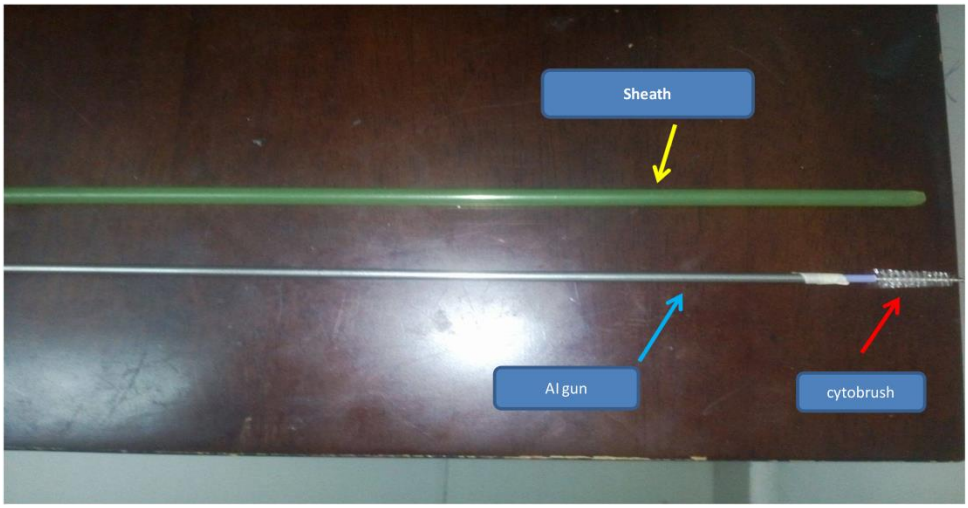

Figure 7.1 : Cytobrush with rod (AI gun) used to obtain endometrial cytological samples.

7.2.4 Statistical analysis

All statistical analyses were performed using the SPSS software (version 18.0, IBM SPSS Inc., Chicago, USA) and Excel 2007. First, numerical data were tested using the Kolmogorov–Smirnov test for normality distribution. The endometritis occurrence was recorded from the clinical examination results of endometrial cytological samples. The agreement between endometrial cytological and ultrasound inspection findings was compared using kappa analyses. Differences were considered significant at $P < 0.05$.

7.3 Results

Results obtained using the CB method showed that six cows (6/53, 11.3%) and five cows (5/53, 9.4%) at 4 and 5 weeks postpartum showed PMN $\geq$ 8%, which is indicative of cytological endometritis (Fig.7.2).

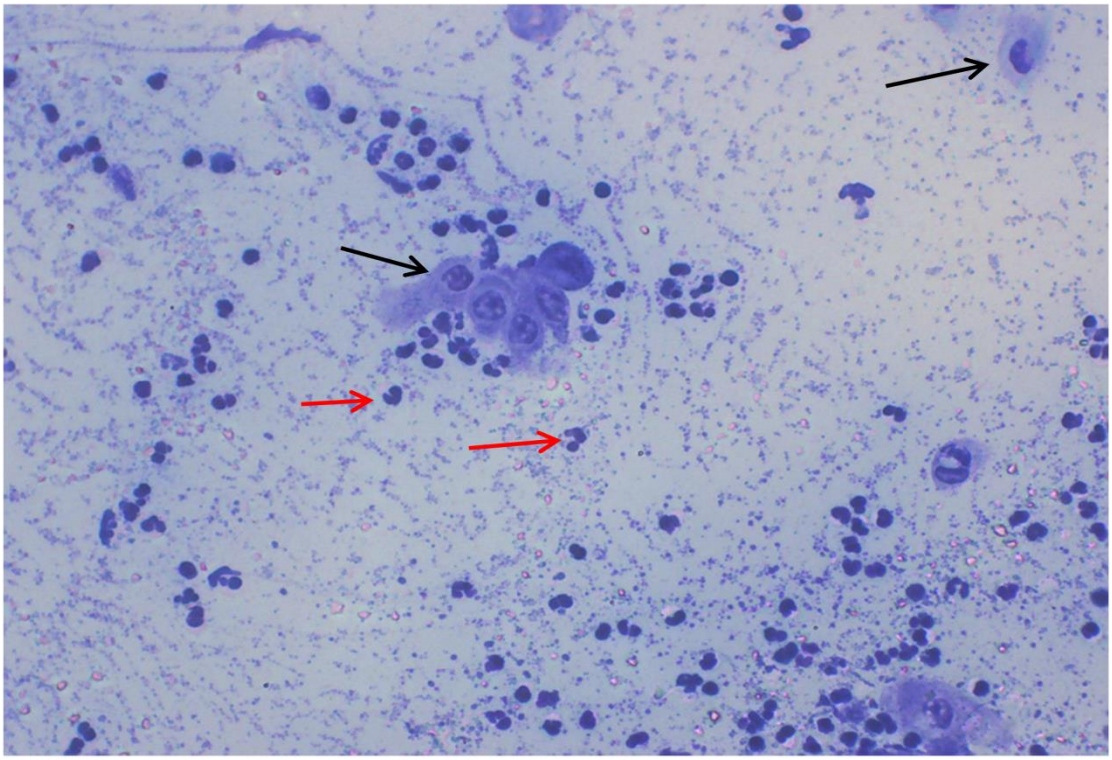

Figure 7.2 : Cytological smear obtained by cytobrush method from subclinical endometritis cow stained with Giemsa stain, Red arrows shows high infiltration of PMN cells (neutrophils). Black arrows pointed endometrial cell (400x).

Ultrasound evaluation 4 weeks postpartum showed that 18 cows had FIU (Table 7.1); this result presented a 0.29 kappa agreement with the cytological method, as well as 83.3% sensitivity and 72.3% specificity. At 5 weeks postpartum, 12 cows were FIU-positive, which translated to a 0.38 kappa agreement, 80% sensitivity, and 83.3% specificity (Table 7.2 and 7.3). According to the CD 4 weeks postpartum, 13 cows showed more than 5 cm and yielded a 0.31 kappa agreement, 66.6% sensitivity, and 81% specificity. At week 5, cervical measurements revealed that seven cows were positive for endometritis, thereby giving a 0.43 kappa value, 60% sensitivity, and 91.7% specificity. When the parameters were combined (FIU + CD) to increase the accuracy of diagnosing endometritis, an improved kappa agreement was observed between ultrasound and CB methods. Consequently, a 0.50 kappa value, 66.6% sensitivity, and 91.5% specificity were obtained at week 4. Similarly, at 5 weeks postpartum, the same comparison resulted in a 0.49 kappa value, 60% sensitivity, and 93.8% specificity (Table7.3).

Table 7.1 : Agreement among diagnostic methods for endometritis at week 4 postpartum.

Week 4 post calving		Cytobrush		Kappa (p= value)
		PMN ≥ 8 %	PMN < 8 %	
Ultrasound (FIU)	Positive	5	13	K=0.29 (P<0.05)
	Negative	1	34	
		Cytobrush		
		PMN ≥ 8 %	PMN < 8 %	
Ultrasound (CD)	**≥ 5**	**4**	**9**	**K=0.31 (P<0.05)**
	< 5	**2**	**38**	
		Cytobrush		
		PMN ≥ 8 %	PMN < 8 %	
Ultrasound (FIU+ CD)	**Positive**	**4**	**4**	**K=0.50 (P<0.05)**
	Negative	**2**	**43**	

Beef cows with percentage of polymorphonuclear neutrophils (PMN%) < 8 healthy and ≥ 8 were endometritis cows by using endometrial cytology at week 4, (FIU) fluid in uterine, a negative (no fluid) or positive (presence fluid) by ultrasonographic evaluation, CD= uterine cervix diameter < 5cm healthy, ≥ 5 cm endometritis cows by ultrasonographic evaluation. The Kappa statistic is a measure of the level of agreement between the tests, where 1 = complete agreement and 0 = no agreement.

Table7.2 : **Agreement among diagnostic methods for endometritis at week 5 postpartum.**

Week 5 post calving		Cytobrush		Kappa (p value)
		PMN ≥ 8 %	PMN < 8 %	
Ultrasound (FIU)	Positive	4	8	K=0.38 (P<0.05)
	Negative	1	40	
		Cytobrush		
		PMN ≥ 8 %	PMN < 8 %	
Ultrasound (CD)	**≥ 5**	**3**	**4**	**K=0.43 (P<0.05)**
	< 5	**2**	**44**	
		Cytobrush		
		PMN ≥ 8 %	PMN < 8 %	
Ultrasound (FIU+ CD)	**Positive**	**3**	**3**	**K=0.49 (P<0.05)**
	Negative	**2**	**45**	

Beef cows with percentage of polymorphonuclear neutrophils (PMN%) < 8 healthy and ≥ 8 were endometritis cows by using endometrial cytology at week 5, (FIU) fluid in uterine, a negative (no fluid) or positive (presence fluid) by ultrasonographic evaluation, CD= uterine cervix diameter < 5cm healthy, ≥ 5 cm endometritis cows by ultrasonographic evaluation. The Kappa statistic is a measure of the level of agreement between the tests, where 1 = complete agreement and 0 = no agreement.

Table 7.3 : Comparison of ultrasound diagnostic techniques using endometrial cytology ≥ 8 %PMN as the reference diagnostic test for endometritis.

	Week 4			Week 5		
Method	Positive/total	Specificity	Sensitivity	Positive/ total	Specificity	Sensitivity
FIU	18/53	72.3 %	83.3 %	12/53	83.3 %	80 %
CD	13/53	81 %	66.6 %	7/53	91.7 %	60 %
FIU+ CD	8/53	91.5 %	66.6 %	6/53	93.8 %	60 %

FIU= fluid in uterine, CD= uterine cervix diameter.

7.4 Discussion

Obtaining the most ideal method to diagnose SCE accurately using high sensitivity and specificity is difficult (Sheldon *et al*., 2006). Numerous studies have investigated factors, such as nutrition, metabolic disorders, uterine infections, and genetic factors, that affect the pregnancy rate in cow herds (Walsh *et al*., 2011); however, other studies used reproductive performance as an indicator to evaluate these diagnostic methods (LeBlanc *et al*., 2002).

This study focused on the comparison between the ultrasound technique and the cytological method in diagnosing of endometritis in beef cows. At weeks 4 and 5 postpartum, the involution of the bovine genital tract was almost complete in healthy cows but delayed among cows with endometritis. The cows with uterine cervix diameters higher than 5 cm after week 4 developed uterine diseases; moreover, these cows may exhibit reduced fertility in the future (LeBlanc *et al*., 2002). Delayed uterine involution and uterine contamination with bacterial species postpartum are associated with uterine fluid accumulation, which is detected by ultrasound examination *(Mateus et al*., 2002) . Our study showed a weak agreement between ultrasound evaluation and the cytological technique, especially at week 4, and a moderate agreement 5 weeks postpartum. These results agreed with previous studies that reported weak agreements between ultrasound measurements of uterine fluids and CB methods in diagnosing cytological endometritis in dairy cows (Barlund *et al*., 2008; Meira *et al*., 2012). These studies explained that both forms of endometritis are diagnosed using these methods; moreover, these endometritis forms include one that is associated with the cellular influx of PMN and another associated with the fluid accumulation inside the uterine lumen; furthermore, a low PMN percentage was observed with decreased uterine clearness (Kasimanickam *et al*., 2005; Barlund *et al*., 2008). In an attempt to evaluate the diagnostic ability, Kasmanickam *et al*. (2004) mixed both the results of endometrial cytology and an ultrasonographic assessment of fluid in the uterus to improve and synergized both sensitivity and specificity.

In our study, both uterine fluid and cervical diameter were useful for detecting affected cows. Results showed improved sensitivity, specificity, and kappa agreement with the cytological method, which is the standard procedure for diagnosing cytological endometritis. This result was verified when the two parameters were combined to diagnose endometritis; consequently, high sensitivity (60%), specificity (93.8%), and 0.49 kappa agreement were obtained. These results were in agreement with those of Barlund *et al.* (2008) and Meira *et al*. (2012), who found that the ultrasound technique is a good, non-invasive, useful, and practical method to estimate uterine fluid or cervical diameter for diagnosing endometritis. Moreover, ultrasound scanning gives quick results and shortening of time compared with other methods used; also, this method may be less contaminated to the uterus compared to uterine cytology and biopsy.

The efficiency of the ultrasound method in the diagnosis of endometritis can be increased when combined with uterine fluids. Such combination yielded a 50% sensitivity, 88% specificity, and a 39% kappa agreement (Meira *et al*., 2012).

7.5 Conclusion

The percentage of beef cows that were positive to cytological endometritis was low (PMN ≥8%) at 4 and 5 weeks postpartum. This study showed that the ultrasound technique is a useful and practical method for diagnosing endometritis during week 4 and 5 of postpartum, especially when combined with detection of intrauterine fluid accumulation and measurement of cervical diameter. This combination resulted in a high sensitivity of 60%, high specificity of 93.8%, and a 0.49 kappa agreement. Although the agreement (Kappa test) of ultrasound examination with cytology method in the current study was not very high (50%), but still the ultrasound method is considered as very effective way as it gives quick onsite field results and can shorten the time need to diagnose compared with other methods. Moreover, ultrasound method may be chosen as it presents less risk of contamination that can jeopardize findings compared with other methods.

CHAPTER 8

GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATION

8.1 General discussion

Based on the results observed in this study, the duration of sample collection for endometrial cytology was shorter (2-3 min.) in CB and CS than LVF (6-10 min.) method. Kasimanickam *et al*. (2005) and Cocchia *et al*. (2012) showed that the time needed to get uterine samples by CB (2-3 min.) was appropriate and quicker than LVF (6-10 min.). Barlund *et al*. (2008) had selected CB as the reference endometrial cytological detection test because it is the most reliable method in the diagnosis of SCE. Besides that, during the CB procedure, only one trained person is needed to get an endometrial cytological sample, whereas, LVF method always requires at least two people to get a uterine specimen.

The results of this study also showed that the mean PMN % at weeks 3 and 4 were higher from the CB method than the other two methods; CS and LVF, respectively. Due to the fiber nature of the cytobrush tip and the rigid nylon, the vertical position of the handle tip allowed more endometrial cells to be picked up from the surface of the uterus in comparison to the other two methods. Besides that, the CB method allows for a deeper penetration of the uterine endometrium resulting in more PMN (Martin-Hirsch *et al*., 2000). Due to the soft nature of the cotton in the CS method, the number of endometrial cells and PMN was lower than in the CB method at weeks 3 and 4 (Cocchia *et al*., 2012). At the same time, the average PMN were lower in cows through LVF method than CB during 15 and 28 days postpartum, which was due to difficulties in getting infused fluid back because the uterus was not fully involuted at this time, consequently only fewer cells were obtained (Kasimanickam *et al*., 2005). The present study showed increased in number abnormal of endometrial cells in LVF compared with CB and CS methods. This is in agreement with previous findings by Barlund *et a*l. (2008), and Kasimanickam *et al* (2005), who believed that LVF method has a harmful effect on the integrity of the cells. Depended on these findings, we used CB as a standard method to collect endometrial samples in following objectives.

The overall occurrence of clinical endometritis (CE) in the present study was 10.97%, which is lower than the range reported by previous studies between 20% to 40% in postpartum dairy cows (Heuwieser *et al*., 2000; McDougall *et al*., 2011). The discrepancy in the results may be due to decreased post-calving problems, such as dystocia, retained placenta, and metabolic disorders. Furthermore, most cows in this study depended on grazing and had low milk production, thereby developing few stress factors and minimal exposure to uterine infection after calving.

E. coli was the common bacteria isolated from healthy (14.6%) and endometritic cows (38.8% in CE cows and 42.8% in SCE cows) in the period between 20 days to 30 days postpartum. The present result agrees with a previous study demonstrating that *E. coli* were the common uterine pathogens in the postpartum period (Sens and Heuwieser, 2013). Although *E. coli* is a common bacteria in the environment, specific strains of this species have been isolated from cows with uterine diseases (Sheldon *et al*., 2006). Endometrial pathogenic *E. coli* is more adherent and invasive in the endometrium compared with *E. coli* isolated from the uterus of clinically unaffected cows. These pathogenic *E. coli* strains develop diseases of endometrial surfaces such as postpartum metritis or endometritis in the bovine genital tract (Sheldon *et al*., 2006). In contrast to most studies, the present study showed a decreased number of *A. pyogenes* (one isolate) in CE cases, leading to few cases of purulent vaginal discharge (scores 2 and 3) (Zobel, 2013). And this result may be due to lower occurrence of dystocia, retained placenta, and postpartum disorders in these cows. However, Sens and Heuwieser (2013) reported in the previous study about bacterial isolation during postpartum period that *E Coli and A. pyogenes* were the dominant bacteria isolated from uterus between 7-24 days postpartum.

The results of antimicrobial sensitivity test in this study showed the inhibition effects of enrofloxacin on the in vitro bacterial growth, most of the isolated bacteria were sensitive to enrofloxacin and tetracycline. This result agrees with finding for another study that concluded in the inhibition role of norfloxacin for E coli (100 %) and A. Pyogenes (75.7 %) (Malinowski *et al*., 2011). However, others studies found the tetracycline the most suitable antibiotic to treat the cases of metritis in cows (Muneer *et al*., 1991; Bhat and Bhattacharyya, 2012). This result also agrees with our finding because tetracycline was the second most effective antibiotic that inhibited bacterial growth in our experiment. Udhayavel *et al*. (2013) reported that a large number of isolated uterine discharge in endometritis cows were sensitive to the ceftriaxone, followed by other antibiotics such as gentamicin, and enrofloxacin Silva and Lobato (1998) found that E coli strains were more sensitive to enrofloxacin, gentamycin. In contrast to our results, one study concluded the failure of using enrofloxacin (1g) to treat the cows with endometritis and combining the enrofloxacin with EDTA-Tris (buffer solution) enhanced getting positive results (Farca *et al*., 1997). However, the present study did not use any antibiotic to treat SCE cows because it was not with study design.

This study showed the effect of calving assistance (dystocia) as a risk factor on CE and SCE, And this due to the severe trauma that occurred in the pelvic canal during calving and consequently uterine contamination in these cows. The results are in agreement with previous studies reporting the effect of calving assistance on the rate of possible uterine contamination after calving (Madoz *et al*., 2013). Rogers *et al*. (2004) mentioned in one study that cows which had dystocia has more chances to suffer from uterine infections and increased culling rate in the future than those that calved without assistance. However, this result is in contrast with another study that did not find any effect of calving assistance on CE and SCE occurrence (Prunner *et al*., 2014a).

In the present study, poor to moderate agreement was found among PMN %, bacteriological findings, and vaginal discharges. According to previous studies, results indicated that abnormal discharges do not necessarily indicate uterine infection (Dubuc *et al*., 2010; Westermann *et al*., 2010). The present study also showed poor agreement between bacterial findings and PMN, similar to the findings of other studies (Barański et al., 2012). Not all of the bacterial species cause inflammation and infusion of PMN to the uterine endothelium because numerous bacteria may be normal inhabitants of the uterus; uterine infection after calving can also be due to other causes, such as yeast and viruses.

Reproductive performance is one of the most common economically important traits in beef production (Bormann *et al*., 2006). The current study showed minimal effects of CE, SCE, and isolated bacteria on the reproductive performance of cows because of the ability of these cows to self-cure; this finding is similar to at reported in a previous study (Prunner *et al*., 2014b). However, most studies showed increased days open and days to conception in cows suffering from uterine diseases, such as CE and SCE (Elkjær *et al*., 2013; Madoz *et al*., 2013). The decreased number of acute infection cases (grade 3) of the endometrium caused by *A. pyogenes* could result in the reduction of the acute destruction of the endometrium layer and impairment of future reproductive performance of these cows. Most of the beef cow herds exhibit high pregnancy rate during the breeding season that they are properly managed (Amundson *et al*., 2006) and uterine contamination can be controlled especially after the resumption of the ovarian cycle (Santos *et al*., 2009a).

The current study also showed a decline in the pregnancy rate of beef cows 45.1 % (37/ 82) at 200 days post-calving. These could be due to several possible factors that affect the reproductive performance of these cows, such as seasonal breeding, prolonged postpartum acyclicity in suckled beef cows, nutrition, breed and uterine infection (Yimer *et al*, 2010). Pregnancy stages on the day 200 after birth were more than 150 days, the reason for that may be these cows have a longer period to overcome their reproductive problems and more chance to be pregnant. Extended post calving anestrus in suckled beef cows is one of the most common limitations to gaining a calf every year (Miller and Ungerfeld, 2008). Climatic stress, parity, extended suckling, nutritional deficiencies, and management practices were the most common reasons for prolonged calving intervals (Diskin and Kenny, 2016).

This study showed that prevalence of subclinical endometritis (SCE) in the beef group was 12.5 %. There were only two studies (Santos *et al*., 2009b; Ricci *et al*., 2015) that have reported SCE in beef cows. According to Santos *et al*. (2009b) study in beef cows, 17% of Angus cows were positive to endometritis by using low-volume uterine lavage method, which greater than our findings in beef cows. This may be because this study used a threshold of 5.5% PMN as an indicator for cytological endometritis and used LVF method to collect endometrial cytology. This study found the ability of beef cows in the postpartum period to clear uterine infection after the resumption of ovarian activity without impairment of fertility

(Santos *et al*., 2009b). In contrast, the second survey by Ricci *et al*. (2015) who included 97 beef cows between 28 and 68 days postpartum demonstrated a 31% prevalence of cytological endometritis with subsequent reduction of reproductive performance. However, The current study showed minimal effects of SCE on the reproductive performance and prolonged anestrus was challenged to beef animals of this study.

The occurrence of SCE in the dairy group in the present study was 15.3%, which is greater than a report of 11.8% by Barlund *et al*. (2008), who used 8% PMN as the threshold during 28 to 41 days postpartum. However, this occurrence was lower than in another study (Denis Robichaud and Dubuc, 2015) where it was reported 36% by using a threshold of 6% PMN at 28 to 42 days after calving. Thus far, no consensus has been established with regard to the effect of threshold value and time of uterine sampling on SCE diagnosis. SCE can be diagnosed using different cut-off values, such as PMN range of 5–18%with either cytobrush or low-volume lavage techniques (Barlund *et al*., 2008).

The prevalence of SCE was little higher in dairy group than beef cows. This may be due to milk production stress factor for these cows in comparison with beef cows. This agrees with previous studies that reported lower SCE cases in beef cows and considered it a minor problem in beef farms and got 90 % of pregnancy rate with optimal management (Santos *et al*., 2009b). The findings also showed that even in beef cows, prevalence of SCE was higher in Brangus breed (18.8%) than K.K cows (4.6%) and this could be due to a genetic difference between the two groups of cows. In addition, the differences could be due to the adaptation of the local breed, the K.K. cows to the hot and humid tropical environment (Perea-Ganchou *et al*., 2005) as compared with the imported Brangus breed.

The study also showed increasing SCE in beef heifers than multiparous cows in both dairy and beef groups, and this may be associated with more incidences of calving problems and postpartum disorders in heifers than multiparous cows.

In the present study, the occurrence of cessation of cycling in dairy cows was 34.6 %. This agrees with a previous study which reported between 11% to 38% of anestrus occurring during 50 to 60 days postpartum (Rhodes *et al*., 2003).

Our finding (34.6 %) of ovarian cessation in the dairy group was higher compared with another study by Yimer (2011) who found 6.1% of dairy cows suffered from ovarian cessation in Friesian cows. The same study showed a higher cessation of ovarian activity occurred in Brangus beef cows (41%) in comparison with K.K breed (Yimer *et al*., 2010) and these were found to be lower than results in this study. This observed difference could partly be explained by the fact that samples collected during this study was during postpartum period (week 5and 7) while the previous studies was achieved based on cows with fertility problems. Our study also agrees

with other earlier results, which included a report where 40% of cows where not cycling (Bartlett *et al*., 1987; Opsomer, 1999). Our results disagree with another study on SCE where lower prevalence of (16 to 20%) anestrus in postpartum cows where reported (Mayer *et al*., 1987). The variation in the prevalence of anestrus among different studies may be because of dissimilar environmental, management conditions as well as differences in energy intake levels at different farms (Bostedt *et al*., 1985; Mwaanga and Janowski, 2000).

The average cessation of ovarian activity in this study was higher in beef cows (81.3%) than dairy cows (34.6%) and most of these cows suffered from inactive ovaries and were having low (1ng/mL) serum progesterone values during 7 weeks of the postpartum period. This may be due to the effect of prolonged suckling in beef cows Another reason can be explained by lesser stress on dairy cows in present study as shown by their low milk production (5-10 kg daily) (yavas and Walton, 2000) compared with the cows of high milk production (30 kg/day) in commercial milk production farms.

The ovarian resumption was higher in multiparous cows in comparison with heifer in dairy group and this result corresponds with other findings of previous studies that revealed multiparous cows were more prone to resume ovarian activity than primiparous cows (Opsomer *et al*., 2000; Santos *et al*., 2009a**;** López *et al*., 2012). This result may be because, most of the heifers suffer from calving problems and postpartum disorders compare with healthy multiparous cows that overcome their postpartum problems and resume ovarian cyclicity during weeks after calving.

Size of ovulatory follicle is considered one of the most common essential factors that affect the reproductive performances in cows. A follicle having a diameter of ≥10 mm is considered as an ovulatory follicle in dairy cows (Sartori *et al*., 2001). However, the transrectal palpation and ultrasound examination of the ovaries of most healthy dairy group showed follicles with diameter 9 mm at week 4 and 10-12 mm at week 5. However, in cows suffering from SCE at four to five weeks postpartum, follicular size was found to be less than 6mm. This agrees with previous study that confirmed effect of uterine infection on the ovarian activity (Williams *et al*., 2007). Furthermore, healthy beef cows in this study had larger follicle diameter than SCE cows during most weeks of this study. However, most of these follicles in the early postpartum period were small in size (less than 5mm) and became atretic follicles, thus making most of the beef cows being in anoestrus (81.3%).

A higher proportion of cows that suffer from uterine infection are often characterized by follicles having small sized diameter and most usually become atretic follicles or become small corpus luteum, after ovulation and will only secrete low levels of the progesterone (William *et al*., 2007) . The first ovulation in the present study was earlier (week 4) in healthy dairy cows compared with SCE cows that occurred in week 7, also ovarian activity was faster in a healthy beef group than SCE group. Our results disagree with a recent study by Gobikrushanuth *et al*.(2016) that revealed, the

period from parturition until the ovary develops large and dominant follicle was not related to endometritis cases in cows. This result may be because of the difference in the intensity of uterine infection among the studies.

The present study showed a delay of resumption of ovarian activity of postpartum cows especially in endometritis beef and dairy cows. The transrectal palpation and ultrasound examination of the ovaries of these cows showed small follicle size, less than 5 mm diameter during the early postpartum period until week 6. These results agree with many previous studies that have demonstrated the effect of uterine diseases after calving on ovarian resumption and dominant follicle development and formation of functional CL (Sheldon *et al*., 2002; Williams *et al*., 2007).

The current study showed increased serum progesterone levels in healthy cows compared with endometritis cows in both beef and dairy groups. These results agree with many previous studies that have reported a significant relationship between uterine health and resumption of ovarian activity after calving (Huzzey *et al*., 2009; Burke *et al*., 2010).

Uterine diseases at early postpartum period inhibit ovarian granulosa cells activity, and the growth of the dominant follicles (Williams *et al*., 2007) and this cause the suppression of estradiol secretion from aromatization of androgens, consequently resulting change in the follicle lifespan and ovulation (Herath *et al*., 2007). In addition, uterine diseases also inhibit LH secretion and affect ovarian activity, which disrupts ovulation process in cows that had endometritis (Sheldon *et al*., 2002).

The SCE group had BCS of 2.5 ($P < 0.05$) compared with healthy dairy cows BCS >2.7. This result agrees with a previous study that reported the occurrence of endometritis cases in cows that were four weeks after calving and usually have thin body condition score (Heuer *et al*. 1999). Most of the cows that suffered serious NEB after calving at the beginning of the postpartum period, exposure to decrease the whole number of neutrophils and lymphocytes compared with cows that suffered from mild NEB (Hammon *et al*., 2006). This study has shown that beef cows with SCE had low BCS after calving and during the ensuing postpartum period as compared with healthy cows. There are several factors that affect the health of postpartum beef cows and these includes postpartum disorders, NEB, suckling calves and poor management. All these factors influence BCS after calving and consequently suffer from SCE, postpartum anestrus, and impairment of the reproductive performance in future.

The cows of the present study tend to lose body weight and body condition scores at the 3rd week of post-calving, especially in the dairy group. One study reported that most of the cows that are have low BCS early after calving have risk factor on their reproductive performance by increasing the interval from calving to conception and pregnancy loss (Santos *et al*., 2009a). This agrees with our results as about 34.6 %

and 80 % of dairy and beef cows respectively had a cessation of ovarian activity during the postpartum period. Thus, all management programs during peri prepartum period to keep body condition score within this score (3.00 to 3.25) are useful to avoid the occurrence of metabolic and uterine diseases and consequently reduce the effect on reproductive performance of these cows (Ribeiro *et al*., 2013). However, this disagrees with our findings, as such, the cows that had a body condition score of 3 at the time of calving, (most of the beef cows) had a prolonged resumption of ovarian activity during the postpartum period. This may be due to differences in breed, and management system between the studies and prolonged postpartum anestrus due to the effect suckling for the beef cows.

The present study showed increased serum levels of BHBA and NEFA in SCE cows in beef and dairy groups during week 3 and 6 postpartum compared with healthy cows and these results correspond with a previous study that reported cows that suffered from SCE and metritis had decreased energy intake and developed increased concentrations of serum NEFA and BHBA until week 4 after calving (Hammon *et al*., 2006). Most the BCS of SCE cows in our study were < 2.5 during 2 and 4 weeks after calving and more prone to NEB and consequently increasing of NEFA and BHBA.

In the current study, the acyclic cows of the dairy group had a lower BCS than cyclic cows during the postpartum period. This agrees with many studies that confirmed the relationship between severe NEB and delayed first ovulation after calving (Wiltbank *et al*., 2006; Santos *et al*., 2009a; Burke *et al*., 2010; Giuliodori *et al*., 2011; Castro *et al*., 2012). Cows that have a high concentration of blood NEFA had increased occurrence of anovulation. Despite the significant relationship between blood level of NEFA, insulin and the duration of the anovulatory period after calving reported (Butler, 2003; Wiltbank *et al*., 2006), other studies did not find any difference in the serum level of insulin and NEFA between healthy, endometritis, ovulatory and anovulatory dairy cows (Krause *et al*., 2014). And this may be explained by that the anovulatory case in dairy cows may be associated more with the inflammatory status than the stress of nutritional conditions that are represented by the degree of the lipid mobilization and the production of milk (Butler, 2003). This explanation, however, does not fully support our findings due to the presence of a high number of healthy beef cows that suffered from prolonged ovarian cessation condition despite these cows were clear from uterine diseases. Maybe because many factors affect the resumption of ovarian activity in beef cows such as calves suckling, breed, parity, environment and management.

The dairy cows with high milk yield are more exposed to the adverse impacts of NEB: however, there is a debate about the effect of high milk production on ovarian activity and a delayed first postpartum ovulation. However, most the dairy cows in the current study had low milk production (5-10 kg/day) and most of dairy group showed resumption of ovarian activity during two months after calving. Most dairy cows of the current study had low milk production in both the healthy and

endometritis cows and the reason for the increased NEFA concentration may be because most of the grazing cows (cows of study), are affected by low dry material intake and maybe this develops decreasing of caloric energy diets that consumed by these cows (Bargo *et al*., 2003).

Generally, the levels of Serum IGF-1 increased progressively with the advance of the postpartum period. Serum IGF-1 concentration of dairy cows was greater than beef cows across w3-w7 of the postpartum period. The reason for this result could be that most of the dairy cows sampled resumed ovarian activity during week 4 of postpartum compared with the beef cows that suffered from prolonged anestrus status. And this agrees with many studies that have demonstrated a clear correlation between blood IGF-I levels and ovarian function in postpartum cows.

Our findings revealed that concentration of IGF-1 was lower in cows with SCE in both dairy and beef cows when compared with healthy cows and this may be due to the effect of inflammation and toxin production by bacteria on hypothalamus-pituitary axis and this could have an effect on ovarian resumption. This result agrees with many previous studies that have reported a significant relationship between uterine health and resumption of ovarian activity after calving (Huzzey *et al*., 2009; Burke *et al*., 2010). Moreover, the same study found an essential relationship between plasma estradiol at the first follicular wave after calving and serum IGF-I (Beam & Butler, 1999). The present study showed increasing estradiol and IGF-I in healthy dairy cows compared with SCE cows and resumption of ovarian activity in most these cows.

Most beef cows tend to have prolonged anestrus postpartum period, In addition to the effect of suckling calves on ovarian activity, NEB that occurs in early postpartum cows also probably played a role because nutritional status (energy and protein intake relative to requirements) partially controls the synthesis and secretion of IGF-I.

Serum IGF-1 is decreases in cows after calving when the requirements of the energy exceed the level of nutrient intake (Spicer *et al*., 1990; Beam & Butler, 1999).

Our study showed increased levels of serum IL-6 and IL-8 during weeks 4-7 postpartum in cows with SCE compared with healthy cows in both type of cattle. This result may be due to an increased immune response, especially in the uterus, and as a result, levels of IL6 and IL-8 in the serum of cows with SCE becomes increased. It is noteworthy that IL-6 is an essential pro-inflammatory cytokine, and plays a vital role in many aspects of the inflammation process like the induction of fever in animals, elevation of vascular permeability and stimulation APPs secretion by the liver (Van Snick, 1990).

One of the most common causes for the increased level of IL-6 is the continuous production by uterine endometrium cells, as a result of the activity of endometrium Toll-like receptors and the recognition of the pathogen that invade the endometrium in postpartum period (Sheldon *et al*., 2009; Turner *et al*., 2012). This reaction is considered the key activation factor to the uterine immune response, which leads to the attraction of leucocyte and enhancement of the activity of neutrophils and macrophages for phagocytosis. Both uterus and peripheral leukocytes could be activated by microorganisms (mainly phagocytes), and could secrete proinflammatory cytokines, contributing to increasing their levels in the uterine lumen (Singh *et al*., 2008).

Many previous studies reported infiltration of neutrophils to the endometrium of cows that suffered from SCE and this was due to exposure the uterus of these cows to many different pathogens microorganisms after calving (Kasimanickam *et al*., 2004; Sheldon *et al*., 2006; Brodzki *et al*., 2015a; 2015b).

Proinflammatory cytokine-like (IL-8), also is considered one of the major neutrophil chemokine, was primitively known as a chemotactic agent produced by activated white blood cells that promote the migration of leukocytes like neutrophils and lymphocytes, have an essential function for inflammatory response and acute and chronic diseases (Tseng and Leibert, 2009). The concentrations of IL-6 and IL-8 in healthy cows started to decrease gradually from week 4 until week 7 in both dairy and beef groups in the current study and this can be explained due to the absence of inflammatory reaction in these cows compared with cows had SCE and were characterized by high levels of proinflammatory agents. Hoffmann *et al*. (2002) concluded in their study that infection caused by viral and bacterial agents may induce rapid immune response and high level of IL8 up to 100-fold. Most the SCE cows of the study suffered from bacterial infection (*E. Coli*) and lead to increasing serum IL-6 and IL-8 in these cows compared with healthy ones.

In contrast, another study revealed that no significant difference exists in the levels of cytokines in serum of healthy and SCE cows. However, a difference in cytokines concentration was noticed in the uterine flush of endometritis cows (Kim *et al*., 2014). Also, a study by Ishikawa *et al*. (2004) did not find a significant concentration of IL-6 difference in both the serum of endometritis and healthy cows. The intensity of infection and immune response in the previous studies may be the reason for the difference with our findings.

The haptoglobin (Hp), serum amyloid A (SAA) are the most frequently demonstrated protein factors in cattle during inflammation (Dowling *et al*., 2002). Our results indicated increased serum SAA and Hp level during most weeks of the postpartum period for both SCE dairy and beef cows compared with healthy cows and this agrees with other studies that have confirmed that APPs, especially Hp, and can be used as a marker to diagnosis uterine inflammatory reaction in dairy cows (Ceciliani *et al*., 2012). The use of Hp as an indicator for diagnosis of SCE is very

useful as there were a few previous studies that included this matter (Brodzki *et al*., 2015a; 2015b).

The level of Hp was higher during 4-7 weeks postpartum in the serum of cows with SCE. These concentrations of APPs may suggest the persistence of chronic inflammation of the uterus from the early postpartum period to day 50 postpartum.

In contrast, Yasui *et al*. (2014), reported that a weak relationship between serum Hp and cytological endometritis, or with decreasing reproductive performance in postpartum cows between 40 and 60 days postpartum. The type of infection and intensity of microbial contamination after calving may affect the immune response, as well as the difference in the breed, age, and environment on the size and type of the immune response in these cows between different studies.

Although the level of both Hp and SAA in endometritis cows was significantly greater than healthy cows, the studies confirmed that Hp is a better indicator of postpartum uterine disease than SAA (Chan *et al*., 2010). This result disagrees with another study which concluded that serum Hp increased in dairy cows with acute metritis, but not in chronic endometritis (Skinner *et al*., 1991). Serum SAA in our findings was gradually decreased with the advance of the postpartum period in both healthy and SCE beef and dairy cows because it is related to initiated immune response (first line immune).

It must be noted that Hp and SAA concentrations are increased in sera of cows subjected to physical stress, such as transportation, exhaustion, starvation, or parturition, during which serum glucocorticoid concentrations increased. All cows of the present study were already checked to ensure these cows are free signs of inflammation and clinical disease to avoid possible errors in the concentration of these proinflammatory and acute phase proteins. Our finding found IL-6, IL-8, and Hp was better markers for postpartum cows that suffer from SEC compared with healthy cows because these factors were increased most weeks of our study.

A portion of the present study was focused on the comparison between the ultrasound technique and the cytological method in diagnosing of endometritis in beef cows. The cows with uterine cervix diameters higher than 5 cm after week 4 developed uterine diseases; moreover, these cows may exhibit reduced fertility in the future (LeBlanc *et al*., 2002). Delayed uterine involution and uterine contamination with bacterial species postpartum are associated with uterine fluid accumulation, which is detected by ultrasound examination (Mateus *et al.*, 2002). Our study showed a weak agreement between ultrasound evaluation and the cytological technique, especially at week 4, and a moderate agreement 5 weeks postpartum. These results agreed with previous studies that reported weak agreements between ultrasound measurements of uterine fluids and CB methods in diagnosing cytological endometritis among dairy cows (Barlund *et al*., 2008; Meira *et al*., 2012). These

studies explained that both forms of endometritis are diagnosed using these methods; moreover, these endometritis forms include one that is associated with the cellular influx of PMN and another associated with the fluid accumulation inside the uterine lumen; furthermore, a low PMN percentage was observed with decreased uterine clearness (Kasimanickam *et al*.,2005; Barlund *et al*., 2008). In our study, both uterine fluid and cervical diameter were useful for detecting affected cows. Results showed improved sensitivity, specificity, and kappa agreement with the cytological method, which is the standard procedure for diagnosing cytological endometritis. This result was verified when the two parameters were combined to diagnose endometritis; consequently, high sensitivity (60%), specificity (93.8%), and 0.50 kappa agreement were obtained. These results were in agreement with those of Barlund *et a*l. (2008) and Meira *et al*. (2012), who found that the ultrasound technique is a good, non-invasive, useful, and practical method to estimate uterine fluid or cervical diameter for diagnosing endometritis.

8.2 Conclusion

Based on the results of this study, the following conclusions are made:

1. The study revealed that prevalence of SCE in beef cows was low (12.5%).
2. Cytobrush method was found to be superior and effective technique to obtain endometrial cytological samples.
3. *E. coli, S. aureus*, and dystocia were the major risk factors found associated with SCE in beef cows.
4. The levels of IL-6, IL-8, Hp, and SAA were higher in cows with SCE in comparison with healthy cows, suggesting their potential role as diagnostic markers for SCE.
5. The resumption of ovarian activity was faster in dairy cows than beef group and also in healthy group compared with SCE one. Prolonged postpartum anestrus was the common cause to increase the period from calving to conception and impair beef reproductive performance.
6. The ultrasound technique is a useful and practical method for diagnosing endometritis during the week 4 and 5 of postpartum, especially when combined with detection of intrauterine fluid accumulation and measurement of cervical diameter.
7. The efficiency of diagnosis of endometriosis in cows increases when we adopt ultrasound examination results with endometrial cytology.

8.3 Recommendations

1. Since this study was achieved using the small population of postpartum cows in Selangor state, more studies are recommended involving all parts of Malaysia.
2. Furthers studies are needed to determine predisposing factor that increases the prevalence of SCE in postpartum cows.
3. The study determined the suitable antibiotic and it to treat cows suffer from SCE by using antibiotic sensitivity test thus further practical studies recommend using these antibiotics in farms.
4. Postpartum anestrus in beef cows is a serious problem thus synchronization programs are needed to enhance reproductive performance of these cows.
5. The study determines the BCS as a risk factor for increased the prevalence of SCE and postpartum anestrus, especially in postpartum beef cows. So maintain these pregnant cows healthy during the prepartum period to avoid NEB after calving.
6. The study is depended on serum interleukins and APPs as a marker for SCE in postpartum cows, so endometrium cytokines and APPs are needed to be tested in other studies.

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APPENDICES

Appendix A

Giemesa stain preparation

Stock solution

1. Dissolve 3.8g of Giemsa powder into 250ml of methanol
2. Heat the solution from step 1 to ~60 °C
3. Slowly add in 250ml of glycerin to the solution from step 2
4. Filter the solution from step 3
5. The solution needs to stand a period of time prior to use. Although times vary based on whom you ask a minimum of two months is usually recommended

Appendix B

Bacteriological part

Blood Agar (BA)

Blood agar base (Dehydrated, HI Media)

Ingredients	Grams/ Liter
Beef heart, infusion form	500.00
Trytose	10.00
Sodium chloride	5.00
Agar	15.00

Final pH (at 25 ℃) 7.3 +0.2

Suspended 40 gm of dehydrated blood agar base in 1000 ml distilled water and sterilized by autoclaving at 15 psi pressure, 121 ℃ temperature for 20 minutes. The molten medium was cooled to about 50 ℃ temperature and aseptically 5% v/v sterile defibrinated sheep blood was added. The above medium was mixed well and poured into sterile petri plates.

MacConkey Agar (MCA) (Dehydrated, HI Media)

Ingredients	Grams/ Liter
Peptic digest of animal tissue	20.00
Lactose	10.00
Bile salt	5.00
Sodium chloride	5.00
Neutral red	0.07
Agar	15.00

Final pH (at 25 ℃) 7.5 +0.2

Suspended 55.07 gm of dehydrate MCA in 1000 ml distilled water and sterilized by autoclaving at 15 psi pressure, 121 ℃ temperature for 20 minutes. The molten medium was cooled to about 50 ℃ temperature and poured into sterile petri plates.

Mueller Hinton Agar (MHA)

Mueller Hinton (Dehydrated, HI Media)

Ingredients	Grams/ Liter
Beef Extract	2.00
Acid Hydrolysate of Casein	17.50
Starch	1.5
Agar	17.00

Final pH (at 25 ℃) 7.3 +0.2

1. Suspend 38 gm of the medium in one liter of distilled water.
2. Heat with frequent agitation and boil for one minute to completely dissolve the medium.
3. Autoclave at 121 ℃ for 15 minutes. Cool to room temperature.
4. Pour cooled Mueller Hinton Agar into sterile petri dishes on a level, horizontal surface to give uniform depth.
5. Allow to cool to room temperature.
6. Check for the final pH 7.3 ± 0.1 at 25 ℃.
7. Store the plates at 2-8 ℃.

Gram stain Preparations

1. The primary stain (crystal violet reagents for staining)

Solution A

2 grams of crystal violet (certified 90 percent of the dye content)
20ml of ethanol (95percent vol/vol)

Solution B

0.8 grams of ammonium oxalate,
80ml of distilled water,
Mix A and B so as to obtain crystal violet staining reagent and store for 24 hours.

2. Mordant (grams iodine)

gram of iodine,
gram of potassium iodide,
300ml of distilled water,

Using a mortar, iodine and potassium iodide are ground, while slowly adding water with continued grinding until all the iodine has completely dissolved. (Store this in an amber bottle)

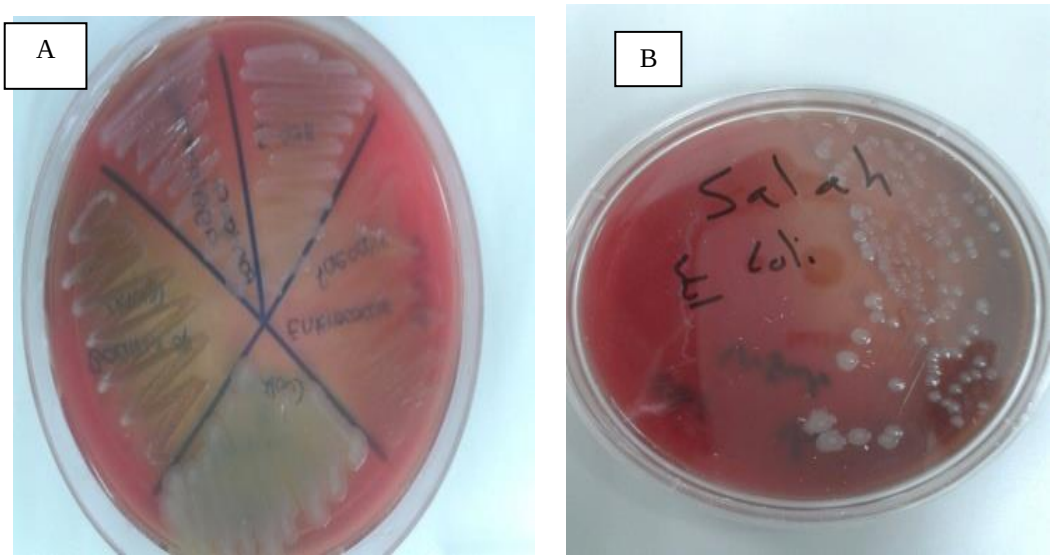
3. Decolorizing agent

Ethanol, 95 percent (vol/vol)
However, acetone or 1:1 acetone with ethanol,
50ml acetone
50ml ethanol (95%)

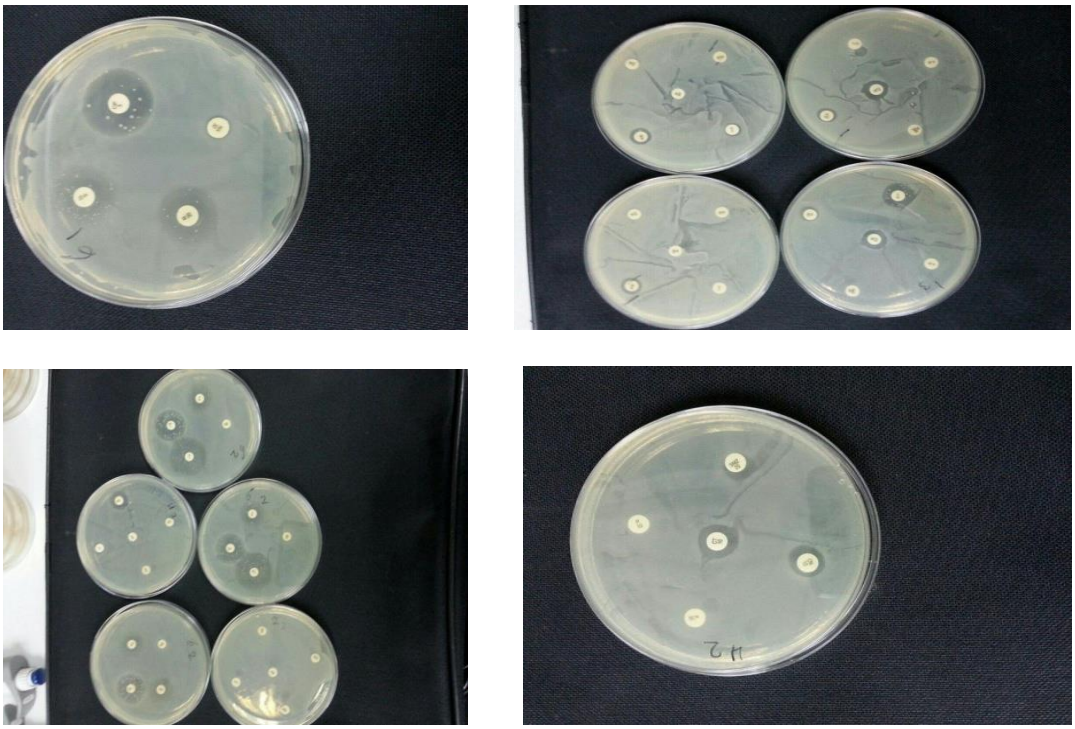
4. Counterstain (safranin)

Working solution:
10ml of the stock solution (2.5 grams Safranin O and 100ml of 95 percent ethanol),
90ml of distilled water,

Transport media used in bacteria isolation (LABCHEM SDN. BHD, MALAYSIA).



Colonies of multiple isolated bacteria species (A) *E coli* isolated bacteria (B) on blood agar.



Antibiotic sensitivity test by using Muller Hinton agar.

Antibiotic disc used in antibiotic sensitivity test

Appendix C

Assay procedure for progesterone using RIA

The antibody coated tubes provided were labeled for standard (S0-S5), total count, non-specific binding, quality control and the samples, 50 µl of the standard solution was added to the labeled tubes mentioned above except the samples tubes; 50 µl of the experimental samples added to the sample tubes. 500 µl of radioactive tracer substance was added into each tube (standard, total count, nonspecific binding, quality control and samples) and were mixed vigorously for 2 minutes and incubated for 1 hour at 18-25 ℃ with shaking (350 rpm). After incubation, the contents were aspirated except the total control and a nonspecific binding tube which was used to measure the sensitivity of the radioactive tracer. The bounding was measured in Wallace wi3ad 1470 automatic gamma. The sensitivity of the assay was the minimal detection limit of the assay which is zero standard and calculated as twice the standard deviation of the zero standards.

Assay procedure for estrogen using RIA

The antibody coated tubes provided were labeled for standard (S0-S6), total count, non-specific binding, quality control and the samples, 100 µl of the standard solution was added to the labeled tubes mentioned above except the samples tubes; 100 µl of the experimental samples added to the sample tubes. 500 µl of radioactive tracer substance was added into each tube (standard, total count, nonspecific binding, quality control and samples) and were mixed vigorously for 2 minutes and incubated for 3 hours at 18-25 ℃ with shaking (350 rpm). After incubation, the contents were aspirated except the total control and a nonspecific binding tube which was used to measure the sensitivity of the radioactive tracer. The bounding was measured in Wallace wi3ad 1470 automatic gamma. The sensitivity of the assay was the minimal detection limit of the assay which is zero standard and calculated as twice the standard deviation of the zero standards.

Appendix D

Operation Steps for ELISA:

1. Set blank wells, standard wells, and test sample wells respectively:

(A) Blank well: do not add samples and horseradish peroxidase (HRP), other operations are the same.

(B) Standard wells: Add standard 50μl to Standard wells.

(C) Test sample wells: Add 40μl of Special diluent and then add 10μl of the sample. (The final sample dilution is five times and the final result calculation should be multiplied byfive times).

(D) Add 50μl of horseradish peroxidase (HRP) into each well, except blank well. Then seal the plate, and gently shake, then incubate 60 minutes at 37 ℃.

2. Discard Liquid excess, drying, fill each well with diluted washing liquid, mix and shake for 30 seconds, discard the washing liquid and tap the plate into absorbent papers to dry, we repeat five times, and then pat dry.

3. Add 50μl of chromogen solution A to each well, and then add 50μl of chromogen solution B to each well. Gently shake and incubate for 10 minutes at 37℃ away from light.

4. Stop: Add Stop Solution 50μl into each well to stop the reaction (the blue changes into yellow immediately).

5. Final measurement: Set blank well zero, measure the optical density (OD) at 450 nm wavelength which should be carried out within 15 minutes after adding the stop solution.

According to standards' concentration and the corresponding OD values, calculate out the standard curve linear regression equation, and then apply the OD values of the sample on the regression equation to calculate the corresponding sample's concentration. It is acceptable to use a variety of software to make calculations.

Appendix E

Ultrasonography

Ultrasound machine used for ovary and uterine examination.

BIODATA OF STUDENT

Salah Noori Mohammed Baba was born on November 1972 in Diyala, Iraq. He pursued his primary and secondary and higher education in Baghdad city. He has got his bachelor degree in veterinary science in 1995, and master degree in theriogenology in 1999 from the faculty of veterinary medicine, University of Baghdad. He worked as a veterinarian at Baghdad Veterinary Hospital for a year, then worked in large cow farms and also in private veterinary clinics for two years in Baghdad, Tikrit, and Kut, Iraq. He worked in the Hashemite Kingdom of Jordan in Sahab and Zarqa state from 2001 to 2005 in dairy cows farms for milk production. He was appointed as a lecturer at the faculty of veterinary medicine, in University of Diyala from 2006 until 2014. He got a scholarship at the Putra University of Malaysia to get a Ph.D. degree in the field of theriogenology and cytology under the supervision Dr. Nurhusien Yimer. Department of veterinary clinical studies, faculty of veterinary medicine, Universiti Putra Malaysia.

LIST OF PUBLICATIONS

N. Salah, N. Yimer, H. Wahid, Y. Rosnina, B. Siti Khairani, M.A. Omar. 2017. Agreement among bacteriological findings, vaginal discharges, and endometrial cytology for endometritis detection in postpartum beef cows. Emirates Journal of Food and Agriculture. 29(5): 396-403.

N. Salah N. Yimer, H. Wahid, Y. Rosnina, A. Khumran, and F. Baiee. Assessment of three different endometrial cytological sampling methods in postpartum beef cows. 2017. The Journal of Animal & Plant Sciences, 27(5): 1515-1521.

N. Salah, and N. Yimer. 2017. Cytological endometritis and its agreement with ultrasound examination in postpartum beef cows. Veterinary World, 10(6).

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