

# **TRANSRECTAL DOPPLER SONOGRAPHY OF OVARIAN STRUCTURES AND UTERUS DURING ESTROUS CYCLE IN DAIRY COWS**

**THESIS**

*By*

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(V-2018-30-009)

**Submitted to**



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*in*

**Partial fulfillment of the requirements for the degree of**

**MASTER OF VETERINARY SCIENCE**

**(ANIMAL REPRODUCTION AND GYNAECOLOGY)**

**DEPARTMENT OF VETERINARY GYNAECOLOGY AND OBSTETRICS**

**2020**



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### **CERTIFICATE I**

This is to certify that the thesis entitled “**Transrectal Doppler sonography of ovarian structures and uterus during estrous cycle in dairy cows**” submitted in partial fulfillment of the requirements for the award of the degree of **Master of Veterinary Science** in the discipline of **Animal Reproduction and Gynaecology** of CSK Himachal Pradesh Krishi Vishvavidyalaya, Palampur is a bonafide research work carried out by **Dr. Tania Soni (Admission No. V-2018-30-009)**, daughter of Smt. Sunita Devi and Sh. Jyoti Soni, under my supervision and that no part of this thesis has been submitted for any other degree or diploma.

The assistance and help received during the course of this investigation have been fully acknowledged.

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**CERTIFICATE II**

This is to certify that the thesis entitled “**Transrectal Doppler sonography of ovarian structures and uterus during estrous cycle in dairy cows**” submitted by **Dr. Tania Soni (Admission No. V-2018-30-009)**, daughter of Smt. Sunita Devi and Sh. Jyoti Soni, to the CSK Himachal Pradesh Krishi Vishvavidyalya, Palampur in partial fulfillment of the requirements for the degree of **Master of Veterinary Science** in the discipline of **Animal Reproduction and Gynaecology** has been approved by the advisory committee after an oral examination of the student in collaboration with an External Examiner.

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## *ACKNOWLEDGEMENT*

Above all, I would like to praise and thank God, the Almighty who has granted countless blessings, knowledge and opportunity so that I can able to finally accomplish the thesis. I wish to express my profound gratitude to God Almighty, for being a symbol of strength, worship and positiveness and thus completing this work. You have made my life magnanimous by always protecting me, helping me with all the difficult times and constantly giving me strength to face everything in life. May the Almighty's name be exalted, honoured and glorified.

It's my genuine pleasure to express my deep sense of thanks and gratitude to my mentor, philosopher and guide Dr. Madhumeet Singh, Professor cum Head, Department of Gynaecology & Obstetrics and chairman of my advisory committee for his dedication. I gratefully acknowledge his support and guidance. Without his thoughtful encouragement this thesis would never have taken shape. I am also grateful to Dr. Amit Sharma and Dr. Parvesh Kumar for their contributions to the direction and richness of this research. I am blessed to have spent time in their tutelage. The experience that I gained from them has nourished my intellectual maturity.

I would also like to thanks other members of the advisory board Dr. P.K. Dogra and Dr. Surender Bansal for their valuable suggestions and useful comments throughout the research work. Their prompt inspiration, timely suggestion with kindness, enthusiasm and dynamism has enabled me to complete my thesis. I would specially like to thank Dr. P.K. Dogra for allowing me to conduct the research and providing assistance requested.

I take pride in acknowledging my gratitude to Dr. Akshay Sharma for sparing his valuable time whenever I approached him and showing me the way ahead. I would like to take this opportunity to express my immense gratitude to all persons who have given their invaluable support and assistance.

I am also equally grateful towards the supporting staff members of the Department of Veterinary Gynaecology and obstetrics Sh. Kamaljeet, Sh. Naresh kumar, Sh. Raman Sood, Sh. Sansar Chand ,Sh. Rakesh Kumar (Special thanks for always motivating and being their unselfishly). A sincere thanks to dairy farm employees and workers, especially Dr Praveen and Mr. Rajkumar for constantly supporting me throughout my research work.

My heartfelt thanks to my seniors (Dr. Akshay, Dr. Ankita, Dr. Pankaj Rana, Dr. Aanchal Rana, Dr. Sadiya Bashir, Dr. Mohit Mahajan, Dr. Ankur Abrol, Dr. Abhay Verma) PG batchmates (Dr. Anushma, Dr. Anshul Chandel, Dr. Isha Sharma, Dr. Sehaj Pal Singh),

juniors (Drs. Ananya, Ankita, Neha, Poppinder, Vivek) for always being there for me and making this journey more memorable. I will always cherish the warmth shown by them. Thanks to all my friends Dr. Shivanshi Walia, Dr. Sukti Naryal, Dr. Anushma Patial, Dr. Anjali Saurab, Dr. Kanika Sharma, Dr. Kanika Singh and many more for their constant support and cooperation. Their timely help and friendship shall also be remembered.

Finally, I acknowledge the people who mean a lot to me, my grandmother, parents and brother for showing faith in me. I salute you all for the selfless love, care, pain and sacrifice you did to shape my life. I would never be able to pay back the love and affection showered upon by my parents. I owe thanks to a very special person, Mr. Paras for his continuous support during my pursuing of M.V.Sc. that made this thesis possible. I greatly value his contribution and deeply appreciate his belief in me. I consider myself the luckiest in the world to have such a lovely and caring family, standing beside me with their love and unconditional support.

Finally, I would like acknowledge to all the personnel who assisted in one way or another towards the accomplishment of this thesis, as well as expressing my apology that I could not mention personally one by one.

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(Signature and Name of the student)

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**Title of the thesis** : Transrectal Doppler sonography of ovarian structures and uterus during estrous cycle in dairy cows  
**Name of the student** : Tania Soni  
**Admission No.** : V-2018-30-009  
**Major discipline** : Animal Reproduction and Gynaecology  
**Minor discipline** : Veterinary Medicine  
**Date of thesis submission** : 19.09.2020  
**Total pages of thesis** : 81  
**Major advisor** : Dr. Madhumeet Singh

**ABSTRACT**

The study was conducted at Instructional Livestock Farm Complex, Chaudhary Sarwan Kumar Himachal Pradesh Agricultural University, Palampur. Ten dairy cows were selected for research and subjected to sonographic examination of ovarian structures from the day of estrus till completion of one estrous cycle using B-mode and color Doppler mode ultrasonography. Developmental pattern of follicles and corpus luteum during two- and three-follicular waves of estrous cycle were studied. The per cent vascularity of corpus luteum during different luteal phases and blood flow to uterus was evaluated based on the analysis of Doppler haemodynamic indices at an interval of 48 hours during an estrous cycle.

Two-wave estrous cycle was more prevalent in dairy cows as compared to three-wave estrous cycle and there was no significant difference ( $p > 0.05$ ) between the diameter of dominant follicle during different waves of two- and three-wave estrous cycle. Corpus luteum diameter and vascularity were significantly higher ( $p < 0.01-0.05$ ) during mid-luteal phase as compared to early and late luteal phase except in three-wave estrous cycle. Corpus luteum vascularity percentage was significantly higher in dairy cows ( $p < 0.05$ ) during late luteal phase in three-wave cycle as compared to two-wave cycle who failed to conceive. Also, there was a significant correlation ( $p < 0.05$ ) between corpus luteum diameter and vascularity irrespective of follicular waves and different luteal phases. A significant difference ( $p < 0.01-0.05$ ) between the recorded haemodynamic indices of middle uterine artery ipsilateral and contralateral to pre-ovulatory follicle was recorded on the day of estrus, however, this difference was found to be non-significant ( $p > 0.05$ ) with the progression of estrous cycle into luteal phase.

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# Chapter 1

## *Introduction*

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Ovarian follicular dynamics is one of the most common important topic in understanding the ovarian physiology, wherein, most of the studies involve European breeds (*Bos taurus*) (Taylor and Rajamahendran 1991a). Research findings regarding follicular dynamics in Zebu cattle (*Bos indicus*) and its crossbreds are limited. The discrepancies in reproductive physiology of Zebu and European breed cows were reported in areas of luteal tissue, dominant follicle diameter, length of estrous cycle and timing of ovulation (Ando *et al.* 2007; Silva-Santos *et al.* 2014). The knowledge of these differences is very crucial for the establishment of appropriate parameters and development of new technologies to increase overall herd reproductive performance (Adams *et al.* 2008; Imron *et al.* 2016).

Ovaries in newborn female calf contain around 100,000 primordial follicles. Follicular growth starts with follicular recruitment defined as entry of small follicle of 3-4 mm diameter into a growing pool in a wave. These wave patterns of follicular growth are documented during pre-pubertal period (Melvin *et al.* 1999), estrous cycle, pregnancy (Taylor and Rajamahendran 1991a) and post-partum period (Murphy *et al.* 1990). One follicle among those selected follicles continues to grow to become dominant follicle (DF), meanwhile rest of the smaller follicles become sub-ordinate follicles (SF) and undergo atresia (Vassena *et al.* 2003; Jaiswal *et al.* 2004; Adam *et al.* 2008). If the growing-phase of dominant follicle coincides with termination of luteal phase, follicle undergoes final maturation followed by ovulation. Most of cattle breeds have 2 and 3 follicular wave pattern in one estrous cycle (Adam *et al.* 2008) but 1, 4 and 5 follicular wave pattern do exist (Viana *et al.* 2000; Bleach *et al.* 2004).

The use of ultrasonography allows better understanding of bovine follicular dynamics in a real time and non-invasive manner (Ireland *et al.* 2000; Herzog and Bollwein 2007). Real time B-mode ultrasonography helps in understanding the physiological, morphological and endocrine changes in estrous cycle of the cows including growth, regression of ovarian follicles, and, changes in corpus luteum (Ireland *et al.* 2000; Kanitz 2001). The knowledge of physiological details of growth and regression of the follicle, corpus luteum (CL), and hormones (Sirois and Fortune 1988) were essentially required for synchronization protocols resulting in acceptable fertility (Fricke and Lamb

2005). Besides this, ultrasonography enables early pregnancy diagnosis, estimation of fetal age and sex, assessment of progression of fetal growth, and, diagnosis of pregnancy disorders. Moreover, fetal sex can also be determined by ultrasonography. The use of ultrasonography in monitoring the response to treatment of ovarian cysts in cattle has also been reported (Viana *et al.* 2013).

In cows, vascular perfusion in the uterine and ovarian artery has been investigated previously using a surgically implanted Doppler ultrasonic probe (Ando *et al.* 2007). However, in recent studies, measurement of uterine blood flow has been made possible with non-invasive color Doppler ultrasonography. Doppler ultrasonography enables imaging of anatomical structure (Herzog and Bollwein 2007) in order to measure physiological and pathological alterations in uterine blood flow (UBF) (Steer *et al.* 1990). This technique has been developed decades ago to measure changes in blood flow during the estrous cycle of mares (Bollwein *et al.* 1998), sheep (Roman-Ponce *et al.* 1983), Simmental cows (Bollwein *et al.* 2000), during pregnancy (Honnens *et al.* 2008), uterine torsion (Hussein 2013), and puerperium (Krueger *et al.* 2009; Heppelmann *et al.* 2013).

Doppler sonography provides real time blood flow visualization from high velocity flow in large vessels to minimal flow in small vessels (Ginther *et al.* 2007). Doppler technology enables the recognition of movement of cells or tissues and is particularly important in the measurement of vascular perfusion. Doppler sonography aids in measurement of blood flow to follicle (Acosta *et al.* 2003; de Tarso *et al.* 2015), Corpus luteum (Miyazaki *et al.* 1998; Acosta *et al.* 2002), before and after ovulation and in uterus (Scully *et al.* 2015). However, two main factors have contributed to the low use of Doppler technology in cattle: 1) the availability and cost of portable devices, and 2) the relative lack of knowledge among practitioners about its potential as a tool to evaluate reproductive function (Viana *et al.* 2013).

Planned study was thus to ameliorate information about increasing the understanding of ovarian physiology as a prelude to effective and efficient utilization in various assisted reproductive techniques like synchronization of estrus, oocyte recovery and transfer of embryo to recipients. The objectives of the study were:

- i. To study development pattern of follicle(s) and corpus luteum during entire estrous cycle in cows via ultrasonography.
- ii. To record blood flow pattern of ovarian structures and uterus during estrous cycle.

## Chapter 2

# *Review of Literature*

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A basic understanding of estrous cycle is necessary for effective reproductive management of cattle as the proportion of cows that become pregnant during a breeding season directly contributes to profitability. Estrous cycle gives heifer or cows a chance to become pregnant after every 21 days. During estrous cycle, follicles develop in a wave like pattern (Driancourt 2001; Miura 2019), which is regulated by changes in the secretion and patterns of different hormones. Following ovulation, the corpus luteum (CL) develops and in the presence of fully developed functional CL, ovulation of other follicles is prevented (Raz and Aharonson 2012; Whitfield 2018).

The application of real-time B mode ultrasonography in bovine reproduction is a recent advancement and has been widely recognized key tool in reproductive management and research. Real-time B mode ultrasonography is a completely non-invasive technique which can be done repeatedly for evaluation of reproductive tract without impairing the normal functioning (Pierson and Ginther 1987; Medan and El-Aty 2010; Whitfield 2018). Therefore, the use of ultrasonography in veterinary practice is emerging rapidly, from its limited use in pregnancy diagnosis, diagnosis of pathological conditions like pyometra, hydrometra or metritis, with high accuracy for planning and performing the reproductive management of the herd. Ultrasonography has also been used for monitoring follicular dynamics, luteal tissue development in the ovaries (Edmondson *et al.* 1986; Adams and Singh 2014; Kleber *et al.* 2017) and uterine echotexture in cattle (Fissore *et al.* 1986; Baez *et al.* 2016). At the time of estrus, uterine lumen is filled with fluids that can be visualized as anechoic area (Pierson and Ginther 1987; Adams *et al.* 2008) whereas other sonographic features of ovaries and uterus such as diameter of follicle, the size of corpus luteum along with its echogenicity and shape and echotexture of uterus can be used as valuable data for diagnosis of stage of estrous cycle (Pierson and Ginther 1988; Schmauder *et al.* 2008; Scully *et al.* 2014).

### **2.1 Estrous cycle in cattle**

Estrous cycle is a series of physiological changes in reproductive tract of bovines and also, accompanied by some behavioral changes. The estrous cycle of cattle starts immediately after puberty. In heifers, puberty occurs at 12-18 months of age or

on attaining the weight of 200-250 kg. The normal duration of estrous cycle in cattle is average 21 days (range 17 to 24 days) (Alvarez *et al.* 2000; Sartoriet *al.* 2004) except for pregnancy, reproductive diseases or other hormonal disorders. During estrous cycle, the ovarian activity follows a cyclic pattern that facilitates cows to come in estrus, show interest in mating and subsequently establishment of pregnancy (Nosier 2003; Raz and Aharonson 2012).

Estrous cycle in cattle follows a well-defined array of events i.e. both physiological and behavioral, which is regulated by hypothalamic-pituitary-gonadal axis (Das *et al.* 2013). Kojima (2003) reported that, the estrous cycle in cattle is divided into four stages i.e. proestrus, estrus, metestrus and diestrus. These four stages have been a part of follicular phase(4-6 days) and luteal phase(14-18 days). The follicular phase i.e. proestrus and estrus, comprises of final maturation and ovulation of the pre-ovulatory follicle while luteal phase i.e. metestrus and diestrus, is the period following ovulation when corpus luteum is the predominant structure on the ovary (Taylor and Rajamahendran 1991b).

### **2.1.1 Follicular phase**

Follicular phase is a momentary phase from progesterone dominance to estrogen dominance. The four major events take place during follicular phase i.e. increase in secretion of gonadotrophin releasing hormone (GnRH), follicular growth, sexual receptivity and ovulation (Evans and Ganjam 2017). Estradiol is the predominant hormone in the follicular phase and is produced itself by follicles in the growing stage. Estradiol 17- $\beta$  is the hormone responsible for initiating estrus and control of the pre-ovulatory LH surge which further results in ovulation. Ovulation is a series of physiological and biochemical changes that lead to rupture of dominant follicles and release of mature oocyte from ovary (Senger 2003).

#### **2.1.1.1 Proestrus**

Proestrus is the first phase of the estrous cycle known as the strengthening phase and lasts for one to three days immediately before the cow comes in estrus. Proestrus usually begins at one or two days after regression of corpus luteum of the previous cycle. Therefore, decline in progesterone concentration is followed by removal of negative feedback and GnRH is released with higher amplitude and frequency (Senger 2003). During proestrus, the ovarian follicles grow under the influence of follicle stimulating hormone (FSH) and increase in luteinizing hormone (LH) causes maturation of growing follicles which results in estradiol secretion (Rahe *et al.* 1980;

Kinder *et al.* 1991; Smith *et al.* 2010; Sakaguchi *et al.* 2019). One of follicle among the cohort becomes dominant and secretes 17- $\beta$  estradiol under the influence of which cattle shows signs of estrus. This 17- $\beta$  estradiol is known to have a positive feedback on hypothalamus and also, there is more secretion of LH from anterior pituitary (Schally *et al.* 1971; Kojima 2003). Estrogen secreted by mature follicles gets absorbed into blood circulation and leads to increase in vascularity (redness of vaginal mucous membrane) and tonicity of uterus (Sumiyoshi *et al.* 2014).

### 2.1.1.2 Estrus

Estrus refers to a period of sexual receptivity during which cows show signs of estrus and become receptive to bulls (Senger 2003). This period lasts for 24-36 hours followed by LH surge at 12-14 hours during which ovulation occurs after its end (Hansel 1961; Busch *et al.* 2008). The duration and intensity of estrus may vary with different breed, parity, milk yield and environment. Rorie *et al.* (2002) studied that there is a direct correlation between intensity of estrus and fertility of cows. Under the influence of gonadotrophic hormones, follicles mature and start secreting 17- $\beta$  estradiol that activates surge center of hypothalamus leading to release of large amount of GnRH and subsequently, LH surge which is responsible for ovulation of dominant follicle (Senger 2003). Varying duration of estrus in cattle as reported by different authors has been shown in Table R1.

**Table R1: Duration (h) of estrus in cattle**

Species/ Breed	Duration of estrus (h)	Author and year of research
Sahiwal cows	15.52 $\pm$ 0.21	Dash (1980)
Zebu cows	13.60 $\pm$ 1.00	Galina <i>et al.</i> (1987)
Zebu cows	7.66 $\pm$ 4.68	Mattonet <i>et al.</i> (1988)
Baoule cows	10.70 $\pm$ 5.10	Chicoteau <i>et al.</i> (1989)
Nellore cows	10.50 $\pm$ 1.00	Pinheiro <i>et al.</i> (1998)
Crossbred cows	34.34 $\pm$ 1.99	Gunasekran (1998)
Beef cows	16.50 $\pm$ 11.90	Floyd <i>et al.</i> (2001)
Holstein Friesian cows	11.8 $\pm$ 4.40	Roelofs <i>et al.</i> (2005)
Ongole cows	15.54 $\pm$ 0.28	Naidu and Rao (2006)
Mithun cows	12.6 $\pm$ 0.30	Mondal <i>et al.</i> (2006)
Sahiwal cows	14.53 $\pm$ 0.80	Layek (2010)
Panganur cows	16.02 $\pm$ 0.74	Ravikiran (2017)

### **2.1.2 Luteal phase**

The luteal phase of estrous cycle consists of three major events i.e. transformation of follicle cells into luteal cells following ovulation (luteinization), growth and development of corpus luteum (diestrus) and luteolysis (lysis of corpus luteum) at the end of luteal phase which occurs under the influence of  $\text{PGF}_2\alpha$  produced by uterine endometrium resulting in decreased concentration of progesterone. The corpus luteum serves as a source of progesterone and it prepares the uterine endometrium for implantation of embryo if fertilization has occurred (Senger 2003).

#### **2.1.2.1 Metestrus**

Metestrus is a phase during which ovulation occurs and it continues for three to four days following estrus. At the time of ovulation, pre-ovulatory follicle ruptures and releases mature oocyte, thereby resulting in formation of red depression on the ovary known as corpus haemorrhagicum. The corpus haemorrhagicum starts growing and secretes progesterone but at this point of time progesterone levels are low (Rahe *et al.* 1980; Forde *et al.* 2010). After ovulation, small and large luteal cells which are derived from theca and granulosa cells respectively are undergoing development to produce progesterone (Smith and Steveson 1995; Sartori and Barros 2011). During this stage, corpus luteum is not fully mature and there are no prostaglandin receptors on it. So, it is usually non-responsive to luteolysis by prostaglandins (Hansel 1961; Miyamoto *et al.* 2009).

#### **2.1.2.2 Diestrus**

Diestrus lasts for 12 to 15 days of estrous cycle. During early diestrus, increases in size and simultaneously, production of progesterone also increases. However, during mid diestrus phase, corpus luteum becomes fully mature, and concentration of progesterone also attains peak (Hansel 1961). The prostaglandin secreted by uterine endometrium binds to luteal cells of corpus luteum and causes breakdown of luteal tissue by local action or by vascular constriction in late diestrus. The prostaglandins also stimulate release of oxytocin from the luteal cells, which acts on the uterus to produce more prostaglandin (Noakes *et al.* 2009). Estrogen from the dominant follicle of second or third follicular wave is also necessary as it induces the uterine prostaglandin synthesis and increases the number of uterine oxytocin receptors. By the end of diestrus phase, lysis of corpus luteum occurs (known as corpus albicans) and negative feedback of progesterone on hypothalamus is removed which results in release

of GnRH and consequently, anterior pituitary produces FSH and LH (Kojima 2003; Miyamoto *et al.* 2009).

## **2.2 Endocrine regulation of the estrous cycle**

Cattle display estrus behavior every 21 days (17-24 days) and the various events during estrous cycle are monitored by the hormones secreted from hypothalamus (GnRH), the anterior pituitary (FSH and LH), the ovaries (progesterone- P<sub>4</sub>, estradiol- E<sub>2</sub> and inhibin) and the uterus (prostaglandins PGF<sub>2</sub>α). These hormones are secreted in a pulsatile manner and function through negative and positive feedback on hypothalamus to regulate the estrous cycle in cattle (Senger 2003).

During the follicular phase of the estrous cycle, the progesterone levels are usually basal due to regression of corpus luteum (CL) (Mann 2009). The increased E<sub>2</sub> concentration is due to rapid proliferation of pre-ovulatory dominant follicle (DF), simultaneous decrease in circulating concentrations of serum progesterone which induces a surge in GnRH and allow the cows to come in estrus and show behavioral signs during which cows/heifers are sexually receptive and stand to be mounted (Frandsen *et al.* 2003; Lynch *et al.* 2010). The pre-ovulatory GnRH surge induces a concomitant FSH and LH surge and this only occurs when serum progesterone levels are basal (Sunderland *et al.* 1994; Mihm and Bleach 2003; Crowe and Mullen 2013).

During metestrus, progesterone levels begin to increase due to formation of corpus luteum in which granulosa cells and theca cells of the ovulated dominant follicle luteinize for the establishment and maintenance of pregnancy or resumption of estrous cycle in absence of fertilization (Nishwender 1981; Kojima 2003; Chasombat *et al.* 2013). In diestrus phase, progesterone concentrations remain elevated and follicular waves continue to grow under the influence of FSH hormone from anterior pituitary. However, the dominant follicle that grows during the luteal phase of the estrous cycle does not ovulate because of progesterone dominance that causes negative feedback on release of LH (Hansel and Convey 1983; Mondal *et al.* 2007; Luo *et al.* 2011; Crowe and Mullen 2013).

## **2.3 Oogenesis and folliculogenesis**

Oogenesis is the process of conversion of oogonia into oocyte. The process of oogenesis starts even before birth of female fetus as the ovarian cortex contains thousands of oocytes already at the time of birth (Van Wezel and Rodgers 1996; Picton 2001; Hafez 2008; Torres *et al.* 2012) that can be classified according to their size, number of granulosa cells and gonadotropin dependence or independence.

Another method of classification is preantral and antral follicles where preantral follicles are further subdivided into primordial, primary or secondary follicles (Hulshof *et al.* 1994; Lucci *et al.* 2002; Mondadori *et al.* 2010). At the antral stage, follicles usually undergo atresia (Hseuh *et al.* 1984; Aerts and Bols 2010) except few reaching the pre-ovulatory stage under gonadotrophic stimulation. As the maturation of oocyte to tertiary stage gets completed, ovulation is achieved and mature oocyte-cumulus complex is released (Paulini *et al.* 2014).

The process of folliculogenesis is a well-organized process and consists of series of events in which a primordial follicle develops to a pre-ovulatory size and simultaneously undergoes growth and differentiation of the oocyte (Aerts and Bols 2010). The ovaries of cows contain large pool of follicles which are at different developmental stages and can be classified into a growing and non-growing pool (Kanitz *et al.* 2001; Orisaka *et al.* 2009). Primordial follicles contain oocyte which is surrounded by flat pregranulosa cells that are present throughout the reproductive life of the cow and are part of a non-growing pool whereas primary, secondary or preantral and tertiary or antral follicles are a part of the growing pool (Senger 2003; Mondadori *et al.* 2010).

The pregranulosa cells of primordial follicle differentiate into granulosa cells which lead to formation of a primary follicle consisting of oocyte and 20-40 granulosa cells (Aerts and Bols 2010). Following maturation, this primary follicle enters into the growing pool from a non-growing pool (Torres *et al.* 2012) and initiation of its growth triggers the further proliferation of granulosa cells, hence, giving rise to the secondary follicle (Fair 2003). Meanwhile, the zona pellucida surrounding the oocyte is still undergoing development and there is increase in number and layers of granulosa cells (Aerts and Bols 2010; Paulini *et al.* 2014). The inner avascular layer of granulosa cells, a layer of theca interna and outer layer of theca externa, makes up the end stage follicle i.e. antral follicle (Milvae *et al.* 1996). The diameter of both follicle and oocyte increases concurrently along with the progression of developmental stages (Torres *et al.* 2012).

#### **2.4 Trans-rectal ultrasonography**

Trans-rectal ultrasonography is a safe, simple and reliable diagnostic tool which utilizes high frequency sound waves that are emitted through transducer and pass through tissue surface. These sound waves after hitting the tissue surface are then reflected back to transducer in the form of echoes and these echoes can be viewed on

the image viewing screen. Linear array transducer contains piezoelectric crystals which are arranged in a row and emits high frequency waves after being energized (Fricke and Lamb 2005; Herzog and Bollwein 2007). These linear array transducers used in bovine reproduction mostly at 5 and 7.5 MHz frequency. Image resolution and depth of tissue penetration by sound waves are inversely related to the frequency of the transducer. A 7.5 MHz transducer results in lesser tissue penetration and greater image resolution and 5.0 MHz transducer results in greater tissue penetration and lesser image detail. Therefore, 7.5 MHz transducer is best for imaging small ovarian structures such as developing follicles and corpus luteum (Fricke and Lamb 2005).

Color Doppler ultrasonography is a recent advancement in the field of bovine reproduction. A higher amount of blood supply to various organs and tissues is directly correlated with its function and hormone production by the ovaries (Acosta *et al.* 2002; Herzog *et al.* 2010; Pinaffi *et al.* 2015). The blood flow measured in uterus and ovaries provide reliable information about genitalia of cows. Uterine blood flow increases near to ovulation and get decreased following ovulation in normal cyclic cows (Ford *et al.* 1979; Acosta and Miyamoto 2004; Acosta *et al.* 2005). During estrous cycle, this technique can be used to evaluate blood flow in growing follicles, corpus luteum and also in differentiating the follicular cyst and luteal cyst (Matsui and Miyamoto 2009). Apart from this, Doppler sonography can be used to visualize early embryonic mortality, during which blood flow to corpus luteum decreases rapidly before the next estrus (Matsui and Miyamoto 2009).

### **2.5 Ovarian follicular dynamics during the estrous cycle**

In cows, growth of follicles occurs in a wave like pattern (Driancourt 2001; Adams *et al.* 2008) during pre- pubertal period (Adams *et al.* 1994; Evans *et al.* 1994; Melvin *et al.* 1999; Atker *et al.* 2010), pregnancy (Taylor and Rajamahendran 1991a; Bo *et al.* 2003; Behzad *et al.* 2010) and estrous cycle (Roche *et al.* 1999; Aerts and Bols. 2010). During an inter-ovulatory period, one, two, three or four follicular waves are observed (Savio *et al.* 1988) where the estrous cycle with three or four follicular waves are slightly longer in duration than cycle with one or two follicular waves. Moreover, the duration of follicle to attain dominance and time from its dominance to ovulation is shorter in three wave cycles (Ahmad *et al.* 1997; Viana *et al.* 2000; Vasenna *et al.* 2003).

### **2.5.1 Follicular growth**

The growth, development and maturation of ovarian follicles are integral processes for high reproductive efficiency in farm animals. Dynamics of follicular growth occurs in two phases, i.e. slow growth phase and fast growth phase (Fair 2003). Initially, it begins with slow growth phase i.e. gonadotrophin independent phase where FSH and LH are not involved in initiation of follicular growth (Buratini *et al.* 2000; McGee and Hsueh 2000; Webb *et al.* 2004). The fast growth phase, gonadotrophin dependent, takes about 5-7 days involves the emergence, growth and selection of dominant follicle followed by either atresia or ovulation (Kastelic 2005).

The rate of growth of small follicles (1-3mm) into larger follicles was known to increase constantly from day 1 to 18 (day 0= day of estrus). The growth of recruited group of follicles had same growth rate in the beginning until one follicle has been selected to become dominant follicle (Burns *et al.* 2005; Adams *et al.* 2008). Gaur and Purohit(2007) recorded that the growth rate of dominant follicle was almost similar during the first, second and third follicular wave of estrous cycle (Imron *et al.* 2016). The energy balance which affects insulin growth factor (IGF), insulin and growth hormone (GH) levels often results in altered pulse frequency of gonadotropins and disturbed follicular growth (Grimard *et al.* 1995; Alvarez *et al.* 2000; Bo *et al.* 2003; Wolfenson *et al.* 2004).

#### **2.5.1.1 Recruitment**

Follicle recruitment is often used synonymously with wave emergence (Ginther *et al.* 1996) and is defined as entrance of cohort of follicles into gonadotropin dependent growing pool (Driancourt 2001). Following ovulation, concentrations of FSH increase which triggers the recruitment of cohort of small follicles of 1-3 mm in diameter (Jaiswal *et al.* 2009). The concentration of FSH hormone attains peak when the future dominant follicle reaches a mean diameter of approximately 4 mm, after which the concentrations starts declining, and remains at basal concentrations till follicular selection occurs (Ginther *et al.* 2000; Celik *et al.* 2005). The process of recruitment takes around 2-4 days and ends by day 4 of estrous cycle in cows (Adams 1999). Following 2-4 days of recruitment of first wave, diameter of follicle starts increasing and reaches a diameter of 6mm which can be detected ultrasonographically (Lucy *et al.* 1992; Defega *et al.* 2016). At this stage of growth, follicles have not attained LH receptors on the granulosa cells (Xu *et al.* 1995).

### 2.5.1.2 Selection

Selection is the process by which one of the follicles from the recruited cohort is selected to become a dominant follicle (Fortune 1994). During the growth of a follicle, there is decline in FSH concentration through the production of estradiol and inhibin (Kastelic 2005). Increase in the concentration of estradiol and inhibin causes a negative feedback on FSH release thereby inhibiting its secretion from the anterior pituitary gland (Sunderland *et al.* 1994; Ginther *et al.* 2001). Therefore, estradiol and inhibin are the main regulators of secretion of FSH (Arai *et al.* 1996; Filho *et al.* 2001; Gaur and Purohit 2007). When the FSH concentration drops down to a basal level, remaining recruited follicles become static and undergo atresia via apoptosis (Ginther *et al.* 2000; Beg *et al.* 2002; Ravikiran 2017). LH receptors are usually present on theca and granulosa cells of healthy follicles at different stage of follicle development (Camp *et al.* 1991; Gaur and Purohit 2019).

In addition to that, presence of LH receptors predominate the follicle to become dominant and be distinguished from other recruited follicles (Bao and Garverick 1998). Follicle selection leads to decrease in number of recruited follicles and the dominant one may undergo ovulation if it belongs to last follicular wave (Ginther *et al.* 1996; Ginther 2000; Gaur and Purohit 2007). At follicular deviation, selected follicle continues to grow and subordinate follicle undergoes atresia. In cows, deviation usually occurs when largest follicle reaches approximately diameter of 8-9 mm (Ginther *et al.* 1996; Ginther *et al.* 2001; Ginther 2016).

### 2.5.1.3 Dominance

The number of recruited follicles is always greater than number of ovulatory follicles as only one follicle continues to grow and reaches a much greater size during each follicular wave. These follicles are known as dominant follicles because after their selection further growth of other subordinate follicles is arrested (Fortune 1994; Fortune *et al.* 2001) and emergence of new follicular wave occurs (Ginther *et al.* 1996; Hadiya *et al.* 2016).

Dominance phase is characterized by presence of one large follicle which is approximately 10-15 mm in diameter and regressing subordinate follicles (Savio *et al.* 1988; Kastelic 2005; Muraya 2013). Following the selection of follicle and establishment of dominance, further recruitment of follicles is inhibited until dominance is lost and ovulation occurs. This inhibition of follicular recruitment occurs under the influence of decreasing FSH concentration (Adams *et al.* 1993; Fortune *et*

*al.*2001). In addition, dominant follicle directly inhibits the growth of small follicles by secreting inhibin and results in atresia. Following atresia or ovulation of dominant follicle, there is a transient rise in circulating levels of FSH and a new follicular wave emerges (Adams *et al.* 1992; Fortune *et al.* 2001; Kleber *et al.* 2017).

Some researchers reported that dominant follicles and the largest subordinate follicle reaches a maximum diameter of approximately 10-20 mm and 8 mm, respectively (Sirois and Fortune 1988; Savio *et al.* 1988; Ginther *et al.* 1989; Jaiswal *et al.* 2009; Imron *et al.* 2016). The size of ovulatory follicle at the time of estrus is directly related with size of corpus luteum forms after ovulation as large sized CL will secrete higher progesterone (Savio *et al.* 1990; Kleber *et al.* 2017; Miura 2019). Also, Perry *et al.* (2005) concluded that pregnancy rates have a direct correlation with size of ovulatory follicle.

The growing follicles are surrounded by network of blood vessels for which the blood flow can be evaluated with Doppler sonography. The blood flow to follicles is similar between medium and large follicles before deviation (Acosta *et al.* 2005) but following selection, blood flow to dominant follicle increases. The blood flow to larger follicle is detectable at the time of deviation as there is a significant difference in blood flow between largest and second largest follicle (Acosta *et al.* 2005; Matsui and Miyamoto 2009).

#### **2.5.1.4 Ovulation of the dominant follicle**

Following standing estrus, the ovulatory follicle ruptures and releases the oocyte. Ovulation occurs at 24 to 32 hours after the onset of standing estrus. During the luteal phase, size of corpus luteum increases along with the production of progesterone, which are usually correlated during period of growth (Miura *et al.* 2015; Ravikiran 2017). The growth of corpus luteum and its ability to produce progesterone depends on its blood supply and various angiogenic growth factors as Fibroblast Growth Factor (FGF) and Vascular Endothelial Growth Factor (VEGF). After the corpus luteum develops to its full size, it enters into static phase (Acosta and Miyamoto 2004).

The regression of the corpus luteum is followed by the secretion of prostaglandins from uterine endometrium (Senger 2003). On release of prostaglandins by uterus, blood flow to corpus luteum increases due to production of nitric oxide (NO) by endothelial and luteal cells. Following vasodilation, there is a release of endothelin and angiotensin which are potential vasoconstrictors and lead to a rapid decrease in blood supply during regression phase (Miyamoto *et al.* 2005). As a consequence, the

production of progesterone also decreases rapidly following decrease in blood supply (Herzog *et al.* 2010; Atkins *et al.* 2010).

### **2.5.1.5 Atresia of follicles**

Atresia of ovarian follicles is promoted by a “programmed-cell death” known as apoptosis. At the time of the first follicular wave, progesterone concentration reaches its maximum and thereby suppressing the LH pulse frequency, which in turn prevents ovulation of dominant follicle (Savio *et al.* 1993; Miura *et al.* 2014; Miura 2019). Follicular regression occurs as a result of reduction in aromatase activity and estradiol synthesis, accompanied by an increased production of insulin-like growth factor binding proteins (IGFBP) which often lead to apoptosis of granulosa cells (Adams *et al.* 1992; Mihm *et al.* 1999; Young and Mcneilly 2010; Makarevich *et al.* 2018).

### **2.5.2 Days of onset of follicular waves**

Follicle stimulating hormone (FSH) plays an important role in regulating emergence and deviation of follicular waves in cattle which can be detected ultrasonographically (Patel *et al.* 2006; Ravikiran 2017). Savio *et al.* (1988) recorded that first and second follicular wave emerges on day 2-4 and day 10-11 in two wave estrous cycle whereas Ginther *et al.* (1989) reported that the follicular waves with follicles of 4-5 mm were first detected on day 0 and 10 for two wave estrous cycle and for three wave estrous cycle, follicular wave emerges on day 0, 9 and 16.

Viana *et al.* (2000) reported that in three wave cyclic pattern, the first wave seen on day  $0.78 \pm 0.44$  of the cycle few time after ovulation (day 0) followed by the second wave emerged on day  $7.11 \pm 1.05$ , with the respective dominant follicle being selected on day  $11.33 \pm 2.06$ , reaching its maximum diameter on day  $12.89 \pm 3.06$  and beginning atresia on day  $15.55 \pm 3.36$ , with intervals among events similar to those observed on the first wave and the third wave (ovulatory) was seen on day  $13.22 \pm 2.44$ . Similarly, in four wave patterns, the first wave emerged on day  $1.25 \pm 0.50$  and followed by intermediate waves on day  $7.25 \pm 0.96$  and  $11.25 \pm 0.96$  of the estrous cycle in three wave pattern. The fourth wave emerged on day  $15.00 \pm 1.41$ , with the ovulatory follicle being selected on day  $18.75 \pm 1.26$ , and reaching its maximum diameter on day  $21.25 \pm 1.71$ . Similarly, Imron *et al.* (2016) reported that in three wave pattern follicular waves emerged on day  $0.4 \pm 0.9$ ,  $8.1 \pm 1.5$  and  $15 \pm 2.1$  of estrous cycle. In four wave estrous cycle, follicular waves were observed on day  $1.4 \pm 1.1$ ,  $7.4 \pm 1.9$ ,  $12.2 \pm 1.5$  and  $16.2 \pm 3.2$  of estrous cycle. However, Nosier (2003) and Siregar *et al.* (2016) reported first, second and third waves on day 0, 8 and 14 of estrous cycle, respectively.

### 2.5.3 Dynamics of second and third follicular wave emergence

The concentrations of estradiol and inhibin drops down following atresia of dominant follicle of first follicular wave, which results in an increase in FSH concentration (Kastelic 2005). As a consequence, there is emergence of new follicular wave and characterized by the selection of the new dominant follicle (Adams *et al.* 1992; Aerts and Bols 2010; Boer *et al.* 2011). Following 6-7 days of the second follicular wave i.e. approximately on 16-17d of the estrous cycle, uterine endometrium starts secreting PGF<sub>2</sub> $\alpha$  (Hansel *et al.* 1975; Quezada-Casasola *et al.* 2014) resulting in regression of CL (Senger 2003; Miyamoto and Shirasuna 2009). Following luteolysis, the decline in the plasma concentration of progesterone, removes the negative feedback of progesterone resulting in reflex increase in GnRH/LH pulse from the hypothalamus and anterior pituitary (McCracken *et al.* 1999; Goff 2004). Increased LH frequency supports growth of the dominant follicle which in turn leads to synthesis of estradiol (Bergfelt *et al.* 1994). The concentration of estradiol and inhibin continues to rise until they attain a sufficient level to cause pre-ovulatory LH surge and subsequently, ovulation (Senger 2003).

In three follicular wave patterns, the dominant follicle of the second wave undergoes atresia whereas the dominant follicle from the third wave ovulates (Aerts and Bols 2010). However, dairy cows with two follicular wave pattern have low fertility as the ovulatory follicle of the second follicular wave is older and contains an aged oocyte in comparison to the ovulatory follicle arising from the third follicular wave (Townson *et al.* 2002; Adams *et al.* 2008). There are many factors such as genetic predisposition, nutrition and surrounding environmental conditions which play an important role in regulating two, three or four follicular wave pattern in bovines (Adams *et al.* 2008). The parturition number also affects number of follicular waves as in *Bos Indicus* cattle the heifers mostly exhibit three follicular waves and cows are known to exhibit two follicular waves during an estrous cycle (Bo *et al.* 2003).

The time period between the appearance of ovulatory follicle and its ovulation is less in two follicular wave patterns as compared to three follicular waves. The maximum size of CL attained during its development is not affected by number of follicular waves as maximum diameter of the ovulatory follicle is almost similar in two and three wave follicular patterns (Jaiswal *et al.* 2009). Mann (2009) reported that there is a positive correlation between size of CL and its ability to secrete progesterone during early stage of its development. Length of estrous cycle reported by various

authors has been shown in Table R2 whereas incidence of two and three follicular wave pattern has been shown in Table R3.

**Table R2: Length of estrous cycle (days) based on number of follicular waves in cows**

Species/Breed	Cycle (Days)			Author and year of research
	Two wave	Three wave	Four wave	
Nellore cows	20.4±0.30	22.80±0.60	-	Ginther <i>et al.</i> (1989)
Dairy cows	20.6±0.50	22.0±0.4	-	Figueiredo <i>et al.</i> (1997)
Dairy cows	20.8±1.7	29.7±4.9	-	Taylor <i>et al.</i> (1993)
Gir cows	19	21.11±1.76	22.25±1.71	Viana <i>et al.</i> (2000)
Native breed of Egypt	19.8±0.6	22.5±0.8	-	Nosier (2003)
Holstein Friesian cows	19	23	-	Sartori <i>et al.</i> (2004)
Zebu cows	21.8±0.8	-	-	Atker <i>et al.</i> (2010)
Thai Native cows	15.50±1.46	21.63±0.51	25.0±1.36	Sakhong <i>et al.</i> (2011)
Kenyan Boran cows	18.6±1.9	23.6±1.05	25.0±4.0	Muraya (2013)
Cross bred	21.50±0.29	22.0	-	Hadiya <i>et al.</i> (2016)
Ongole Cattle	22.0±1.6	-	22.8±2.9	Imron <i>et al.</i> (2016)
Panganur cows	20.25±0.48	21.27±0.50	-	Ravikiran (2017)

**Table R3: Incidence (%) of two and three follicular wave pattern in cows**

Species/Breed	Follicular waves		Author and year of research
Girolando Cattle	Two waves: 62.5% Three waves: 37.5%		Filho <i>et al.</i> (2001)
Native breed of Egypt	Two waves: 71.4% Three waves: 28.6%		Nosier (2003)
Bos Indicus cows	Two waves: 78.57% Three waves: 21.42%		Gaur and Purohit(2007)
Thai heifers and cows	Heifers:	Two waves: 38.10% Three waves: 47.62%	Sakhong <i>et al.</i> (2011)
	Cows	Two waves: 17.24% Three waves: 82.76%	
Kenyan Boran Cows	Two waves: 25.6% Three waves: 66.6% Four waves: 6.67%		Muraya (2013)
Crossbred cows	Two waves: 66.6% Three waves: 33.3%		Hadiya <i>et al.</i> (2016)
Ongole	Three waves: 66% Four waves: 34%		Imron <i>et al.</i> (2016)
Panganur cows	Two waves: 25% Three waves: 75%		Ravikiran (2017)

#### 2.5.4 Relationship between size of pre-ovulatory follicle and corpus luteum

The size of pre-ovulatory dominant follicles varies according to number of follicular waves during the estrous cycle (Celik *et al.* 2005, Lopes *et al.* 2007) as dominant follicle of third and fourth wave gets less time to reach pre-ovulatory capacity. The growth and development of CL is directly correlated with pre-ovulatory dominant follicle having adequate number of granulosa cells and LH receptors on theca and granulosa cells (McNatty *et al.* 1979; Lynch *et al.* 2010; Ishak *et al.* 2017; de Tarso *et al.* 2017). Vasconcelos *et al.* (2001) and Rahman *et al.* (2012) reported a positive correlation between size of pre-ovulatory follicle and diameter of CL as it forms from luteinization of granulosa and theca cells of ovulatory follicle. The number and size of follicles on ovary of cows depends on season of the year and reproductive stage (Godoi *et al.* 2002; George *et al.* 2005; Mossa *et al.* 2012). Busch *et al.* (2008) also reported that CL resulting from pre-ovulatory follicles having a diameter of 10 to 12.5 mm secretes less progesterone in comparison to follicles having diameter greater than 15mm. The size of ovulatory follicle and diameter of CL has been shown in Table R4.

**Table R4: Size (mm) of preovulatory follicle and diameter of corpus luteum in cows**

Size of pre-ovulatory follicle (mm)	Diameter of CL (mm)	Author and year of research
11.49±0.48 13.57±0.35	20.79±1.06 24.17±0.83	Pfeifer <i>et al.</i> (2009)
14.1±0.3 12.2±0.03	15.7±0.7 13.3±0.4	Pandey <i>et al.</i> (2011)
13±0.2 12.7±0.13	17.5±0.3 17.1±0.3	Pinaffi <i>et al.</i> (2015)
12.24±0.71 12.30±0.22	18.94±0.47 19.44±0.87	Imron <i>et al.</i> (2016)
9.4±0.47 8.33±0.38	15.52±0.99 15.02±0.51	Ravikiran (2017)

#### 2.6 Doppler ultrasonography of reproductive tract

The vascular perfusion to uterus and ovaries are crucial for supply of nutrients, hormones and growth factors for development of female genital tract. Doppler sonography is considered as most potential tool for determination of arterial blood flow in humans and animals (Honnens *et al.* 2008). Uterine blood flow is measured using Doppler indices namely: the pulsatility index (PI), the resistivity index (RI), the blood flow volume (BFV), the time averaged maximum velocity (TAMAX) and time averaged mean velocity (TAMEAN). It has been studied that variations in levels of

estrogen and progesterone hormone are responsible for rhythmic changes in blood flow in cows and mares (Ando *et al.* 2004).

### **2.6.1 Doppler indices of middle uterine artery**

The middle uterine artery (MUA) originates from anterior division of internal iliac artery and moves towards the direction of uterus. The middle uterine artery further branches to supply the uterus (arcuate vessels), ovaries (ovarian branches), vagina (vaginal branches) and fallopian tubes (Ando *et al.* 2004; Hassan *et al.* 2017). Trans-rectal color Doppler ultrasonography is a highly efficacious technique to measure blood flow through middle uterine artery (Honnens *et al.* 2008). Blood flow to middle uterine artery during estrus, inter-ovulatory period and pregnancy has been studied using color Doppler ultrasonography (Magness *et al.* 2000; Ginther *et al.* 2007; Arashiro *et al.* 2013).

The vascular perfusion to uterus and ovaries is directly under the influence of steroid hormones concentration (Dickey 1997; Bollwein *et al.* 2000; Hawkes *et al.* 2016). Necessarily estrogen acts as potent vasodilator but the mechanism of intracellular signaling for this effect is not fully known (Greiss *et al.* 1986; Chen *et al.* 2004; Bollwein *et al.* 2016). During estrus and ovulation, estradiol (E<sub>2</sub>) serum concentration is known to be higher, which bring about increased production of endothelial nitric oxide and results in vasodilation (Acosta *et al.* 2003). However, under farm conditions, there are several factors which affect uterine blood flow such as thermal stress, parasitism, nutritional deficiency and poor management (Roman-Ponce *et al.* 1978; Moonmanee *et al.* 2018). Blood flow measured in uterine arteries is reflected by PI (pulsatility index), RI (resistivity index), TAMV (time averaged mean velocity) and TAMAX (time averaged maximum velocity) (Honnens *et al.* 2008; Sprague *et al.* 2009; Hawkes *et al.* 2016).

#### **2.6.1.1 Resistivity index**

Resistivity Index (RI), also known as Pourcelot's ratio, is suggestive of tissue perfusion during the diastole (Singh *et al.* 2016). In other words, it can be expressed as ratio of systole to diastole peaks and calculated as,  $RI = (A-B)/A$ , where A and B are the systolic and diastolic peaks (Thompson *et al.* 1988). The higher value of RI is reported when distal vascular bed constricts and conversely, a low value of RI indicated decreased impedance to blood flow in the distal vascular bed (Thompson *et al.* 1988; Silva 2011). Bollwein *et al.* (2000) reported lower values of RI during

proestrus stage of estrous cycle. Hassan *et al.* (2017) also reported lower RI on the day of estrus which is indicative of greater vascular perfusion to uterus.

During luteal phase, RI is usually higher which is suggestive of decreased blood flow to reproductive system due to higher plasma progesterone concentration (Ford *et al.* 1982). Hassan *et al.* (2017) also reported higher RI on day 10 of estrous cycle (diestrus) followed by a decrease after Day 18. After day 18, a decline in RI can occur due to decrease in plasma progesterone concentration and an increase in estradiol levels (Meinecke *et al.* 1987; Debertolis *et al.* 2016).

During the luteal phase, predominant sex hormone is progesterone (P<sub>4</sub>) and being the potent vasoconstrictor, it intensifies the impedance to uterine blood flow due to which uterus remains inactive. Barbagallo *et al.* (2001) recorded a positive correlation between RI and P<sub>4</sub> during luteal phase. However, no correlation between plasma P<sub>4</sub> concentration and resistivity index was reported by Bollwein *et al.* (2000). Also, negative correlation was found between RI and P<sub>4</sub> in cows (Ford and Chenault 1981) and ewes (Ford *et al.* 1982; Panarace *et al.* 2006; Elmetwally 2016).

#### **2.6.1.2 Pulsatility index**

The pulsatility index demonstrates the resistance in the vascular bed distal to the site of measurement. Pulsatility Index (PI) is negatively correlated with the vascular perfusion and thus, the increase of PI in the uterine arteries might be due to vasoconstriction of the blood vessels at the point of measurement (Elmetwally and Meinecke-Tillmann 2012; Elmetwally 2016). The Pulsatility Index (PI) can be measured as  $PI = (A-B)/M$ , where A and B are systolic and diastolic peaks whereas M stands for mean maximum Doppler-shifted frequency over the cardiac cycle (Thompson *et al.* 1988). The pulsatility index is inversely correlated with vascular perfusion and decrease in its value is followed by an increase in uterine blood flow (Honnens *et al.* 2008)

According to Hassan *et al.* (2017), the higher PI values were reported on Day 10 when compared with values of PI on Day 0 and -1 i.e. estrus and proestrus, respectively. Meanwhile, Moonmanee *et al.* (2018) reported significantly lower RI and PI on ovulatory side uterine artery as compared to contralateral middle uterine artery. Beef cows that failed to ovulate following proper synchronized ovulation protocol were found to have lower uterine artery BFV but they have higher PI values than those of ovulating cows. Therefore, it can be concluded that increased impedance to uterine

artery blood flow during pre-ovulatory period leads to decreased chances of ovulation in *Bos indicus* beef cows (Debertolis *et al.* 2016).

### 2.6.1.3 Velocity indices

The hemodynamic variables related to the acceleration of the blood flow to the uterus (TAMEAN and TAMAX) increased during high plasma estrogen concentration, similar to that observed during estrous cycle (Bollwein *et al.* 2000). The uterine blood flow is controlled by both estradiol and progesterone hormone (Ando *et al.* 2004; Debertolis *et al.* 2016), therefore, TAMEAN values during the follicular phase were higher than in luteal phase in both the uterine arteries (Ando *et al.* 2004). Low values of TAMEAN was observed on Day 0 (day of ovulation) and Day 1, whereas highest value of TAMEAN were observed between day -3 and -1 of estrous cycle, respectively (Debertolis *et al.* 2016). Bollwein *et al.* (2000) also reported a positive correlation between TAMEAN and estrogen concentrations and negative correlation with plasma progesterone levels.

Rawy *et al.* (2018) studied that following estradiol benzoate administration in cows, led to a significant increase in TAMEAN, BFV and diameter of uterine artery. Thus, it is proved that changes in the uterine blood flow associated with the estrous cycle are directly correlated with plasma concentration of estradiol (Bollwein *et al.* 2000).

### 2.6.1.4 Blood flow volume

Blood flow volume= TAMEAN [cm/s] x  $\pi$  x (D [cm]  $\times 0.5$ )<sup>2</sup> x 60, where TAMEAN was the timeaveraged mean velocity of blood flow in the uterine artery (Blanco 2015). Waite *et al.* (1990) reported a higher uterine blood flow at estrus as compared to the luteal phase of the cycle. The BFV values were positively correlated with blood flow velocities, DF growth rates and E<sub>2</sub> concentrations in the ovulating cows. Hence, it is indicative of the importance of uterine blood flow, follicular growth, and E<sub>2</sub>-vasodilation during pre-ovulatory phase in the ovulatory cycle of *Bos indicus* beef cows (Moonmanee *et al.* 2018). Sharma *et al.* (2019) also reported higher BFV, TAMAX and TAMEAN in both the middle uterine arteries of endometritic cows as compared to non-endometritic cows owing to vasodilation of middle uterine artery following endometrial inflammation. Similarly, after parturition, uterine blood flow decline tremendously, especially during the first week after birth due to decrease in E<sub>2</sub> levels (Herzog and Bollwein 2007).

### **2.6.2 Relationship between vascularity of follicle, corpus luteum and progesterone secretion**

Changes in ovarian blood flow are closely associated with ovarian physiology such as secretion of hormones and various vasoactive peptides (Acosta and Miyamoto 2004; Miyamoto *et al.* 2009). Also, follicular growths are closely related to blood flow to follicles, whereas reduced vascularization to thecal layer is related to follicular atresia (Matsui and Miyamoto 2009; Gaur and Purohit 2019). Therefore, assessing blood flow to follicles can be considered as an important diagnostic tool in cattle reproduction.

Color Doppler ultrasonography has been successfully used for evaluation of follicular haemodynamic profiles in cows (Siddiqui *et al.* 2009a). Increase in blood flow to follicle is a possible indicator of increased ovulatory capacity of pre-ovulatory follicle in heifers (Siddiqui *et al.* 2010; Arashiro *et al.* 2013). The size and blood flow to pre-ovulatory follicle had a positive impact on size and vascularity on subsequent CL and its ability to produce progesterone (Bollwein *et al.* 2010). Previous studies have reported that increase in blood flow to pre-ovulatory follicle have hastened the transport of nutrients, gonadotrophins and other components important for follicle maturation and ovulation (Acosta and Miyamoto 2004). Similarly, greater supply of blood, hormones and nutrients had a positive effect on granulosa and theca cells as it enhances the luteinization process and productivity of CL (Miyamoto *et al.* 2009).

In mares, blood flow seen within the follicular wall has been closely associated with the maturity of pre-ovulatory follicle and quality of oocyte (Ginther *et al.* 2007). In addition to that, there is a positive correlation between amount of blood perfusion to pre-ovulatory follicle, its size and fertility in heifers (Siddiqui *et al.* 2009b) and mares (Silva *et al.* 2006). So, small sized pre-ovulatory follicle and low progesterone production may be directly correlated with sub-fertility in cows. Therefore, development of color Doppler ultrasonography has made an easy and accurate evaluation of vascularity and quality of developing CL to detect subsequently pregnant and non-pregnant cows (Herzog *et al.* 2011).

### **2.7 Steroids concentration**

Follicular development during estrous cycle is directly under the control of gonadotrophic hormones (Lucy *et al.* 1992; Nosier 2003; Chasombat *et al.* 2013). Plasma estrogen and progesterone levels keep changing during estrous cycle and these changes bring about change in hypothalamic-pituitary ovarian axis that is basis for

regulation of estrous cycle (Pineda 2003). The hormonal profile is characterized by increase in serum concentrations of estradiol and progesterone during different stages of estrous cycle.

### **2.7.1 Estrogen**

The concentration of estradiol increases simultaneously with presence of estrogen secreting active follicle on the ovary (Shimon *et al.* 2001). The increase in serum concentrations of 17- $\beta$  estradiol released from ovary during estrus corresponds to LH pulses which occur during early luteal phase (Walters *et al.* 1984; Senger 2003). The estrogen concentration decreased significantly from day 0 to day 3 of estrous cycle due to ovulation and subsequent development of luteal tissue under the influence of luteinizing hormone (Hafez 2008; Noakes *et al.* 2001). The concentration of estrogen increases slightly from day 3 to day 6 but do not attains peak which is indicative of development of dominant follicle of first wave (Alvarez *et al.* 2000). Following atresia of a dominant follicle, the levels of estrogen decrease again.

The production of estradiol from pre-ovulatory follicle depends on ability of theca interna cells to produce androstenedione and enhanced ability of granulosa cells to convert androstenedione to estradiol. Estradiol further induces expression of FSH and LH receptors in granulosa cells for stimulatory action of FSH hormone on aromatase activity (Perry *et al.* 2014).

Nosier (2003) reported that the 2 and 3-wave period showed positive correlation between follicular size and estradiol concentration and also indicated that ovulation occurs only when estradiol levels attain peak ( $5.95 \pm 0.70$  pg/ml in 2 wave and  $5.23 \pm 0.29$  pg/ml in 3 wave period). Perry *et al.* (2014) stated that in all cows exhibiting signs of estrus, a significant positive correlation was found between diameter of follicle and estradiol peak concentration, but no such correlation was found between diameter of follicle and concentration of estradiol in cows not exhibiting estrus. Similarly, the estradiol concentrations in ovarian follicular fluid have been found much higher in large sized follicles as compared to medium and small size follicles (Mondal *et al.* 2004).

### **2.7.2 Progesterone**

Following ovulation, the theca and granulosa cells of ovulated follicle luteinize and start secreting progesterone due to formation of corpus luteum. During the diestrus phase of estrous cycle, the follicular waves continue under the influence of FSH but the dominant follicle do not ovulate (Hafez 2008; Diaz *et al.* 2002) due to elevated levels

of progesterone. The progesterone through negative feedback allows LH pulses of greater amplitude but of lesser frequency that is inadequate for ovulation. The progesterone concentration begins to rise for establishment and maintenance of pregnancy but known to drop 3 days before next estrus (Nishwender 1981; Young and Mcneilly 2010; Atkins *et al.* 2013).

The progesterone concentration increases significantly from Day 0 to 15. This continuous increase in progesterone concentration upto day 18 occurs due to functional activity of corpus luteum followed by decrease in concentration due to initiation of luteolysis (Hafez 2008). The concentration of progesterone is also influenced by nutritional status. The pattern of progesterone in milk has been found similar as that in plasma, although the concentration is much higher in milk than those of plasma (Mondal *et al.* 2007). The concentration of progesterone at the time of estrus in milk was 0.5 ng/ml, increase to 18 ng/ml on day 15 of estrous cycle and decline 3 days before next estrus (Mondal *et al.* 2004).

In less than a decade, the large amount of new information has been generated through the use of diagnostic ultrasound in the field of bovine reproduction. The ultrasonography has contributed incredibly as it has shown that cattle exhibit 2 or 3 waves of follicular development throughout the estrous cycle. Besides this, ultrasonography also enables monitoring of growth of individual follicles over time and thus, made understanding of follicular wave pattern a bit easier. Doppler sonography is a recent advancement in bovine reproduction which includes evaluation of genital blood flow. The ovarian and uterine blood flow vary according to day of estrous cycle, ovulating ovary, growth of ovulatory follicle and the stage of corpus luteum development. The use of ultrasound has broadened the scope to increase our understanding of bovine reproduction and manipulate the reproductive processes to maximize the reproductive efficiency of this species.

## Chapter 3

### ***Materials and Methods***

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Present study involves the evaluation of uterus and ovarian structures during the estrous cycle using Doppler sonography in dairy cows. The objectives of the study were to carry out sonographic examination of ovarian structures: follicle and corpus luteum (CL) and uterus, from the day of estrus and thereafter, at 48 hours interval using B mode and color Doppler mode ultrasonography. The study involved recording of number of follicles within the scanned ovary, measurement of follicular diameters, timing of ovulation, recording of the CL diameter and blood flow to uterus and corpus luteum. Cows were assessed for length of estrous cycle, number of follicular waves, development patterns of follicles and corpus luteum. The research was conducted in collaboration with Instructional livestock Farm Complex (ILFC), CSK Himachal Pradesh Krishi Vishvavidyalaya (CSKHPKV), Palampur (32.6°N, 76.3°E, altitude 1290.8m) during the period of March, 2019 to December, 2019.



**Plate 1: Instructional livestock Farm Complex (ILFC), CSK Himachal Pradesh Krishi Vishvavidyalaya (CSKHPKV), Palampur**

#### **3.1 Experimental cows**

Ten cows (Jersey crossbred and Holstein Friesian; n=10) kept under standard feeding conditions, under natural light, housed in dry concrete sheds and milked twice daily were selected for the study. Cows were clinically healthy and normal cyclic along with no history of reproductive abnormality.

##### **3.1.1 Care and management of cows**

Cows were maintained under loose housing system with proper facility of airing with cooling fans. Depending upon seasonal availability and economic as well as social status of farm, all cows were provided with ad-libitum green and dry fodder. Cows were allowed to graze in pastures from 07:00 am to 12:30pm. Concentrate feed and mineral mixture was also supplemented to meet the maintenance and production needs. These cows also had free access to water. Cows were screened for tuberculosis at regular intervals. All the cows were vaccinated against Haemorrhagic septicemia (HS), Black Quarter (BQ) and Foot and mouth disease (FMD) according to the advised schedule followed at dairy farm. Deworming was also done every 6 months for the control of endo- and ecto-parasites. The animal sheds were properly cleaned twice a day by farm workers. All the cows were kept under uniform farm conditions with feeding as per the standards given by National Research Council (NRC 2001).

### **3.1.2 Estrus detection**

All the cows were observed for the behavioural signs of estrus such as mounting, standing still while being mounted by the other cows, bellowing, increased activity, vulvar edema and cervico-vaginal discharge hanging from vulva. Following observation of behavioural signs of estrus, cows were examined per rectally. Cows with normal genitalia, clear cervico-vaginal discharge and good uterine tone were selected for the study. All selected cows were inseminated twice daily according to AM-PM rule (Dransfield *et al.* 1998) and the insemination was done with French semen straws from sperm station of Himachal Pradesh Livestock Development Board, Palampur.

## **3.2 Ultrasonographic examination**

### **3.2.1 Trans-rectal ultrasonography of ovaries**

Trans-rectal ultrasonography of the ovaries and uterus was started on the day of estrus and thereafter, at 48 hours interval to study development pattern of follicle(s) and corpus luteum during estrous cycle in cows using Brightness mode (B- mode). Also, blood flow of ovarian structures was recorded using color Doppler and spectral mode.

The portable ultrasound machine (Mindray Z5; VETMODEL 75L50EAV; Plate 2) equipped with linear rectal transducer of 7.5 MHz frequency was used to scan ovarian structures. The physiological status of follicles (e.g., growing, sub-ordinate, dominant and regressing) and corpus luteum present on both the ovaries was examined. The dimensions of ovarian structures were recorded and correlated with the previous examinations to know about pattern of development of follicles and CL. All the findings and various echographic images were saved for further analysis.



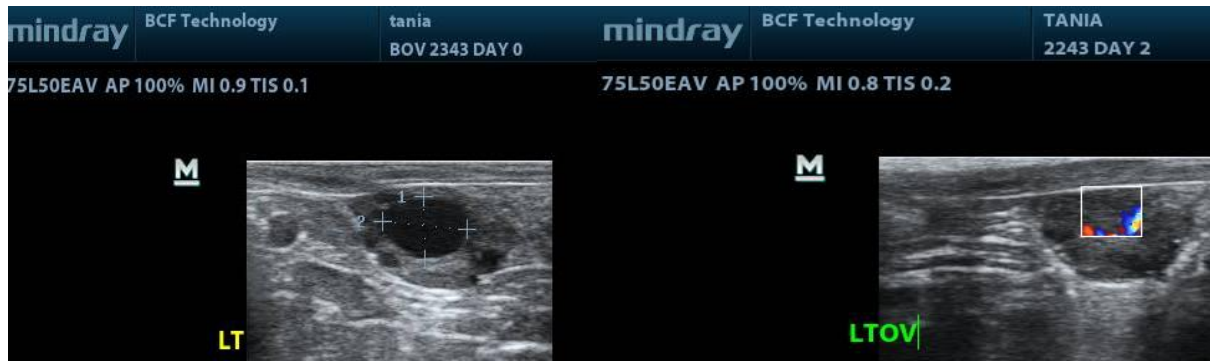
**Plate 2: Portable ultrasound machine (Mindray Z5; VETMODEL 75L50EAV)**

### **3.2.1.1 Procedure**

Prior to ultrasonography, the cows were properly restrained and faeces were manually removed from rectum in order to get clear ultrasound scans for better diagnosis. To facilitate smooth and painless insertion of transducer through anal sphincter, both transducer and sleeved arm were properly lubricated with obstetrical lubricant. Preliminary examination of genital tract was done before the introduction of linear rectal probe. Transducer was placed proximal to the genital tract and moved slowly towards to cranial side. Estrus was confirmed by presence of dominant follicle on one of the ovaries, regressed CL of previous cycle with no or little blood supply, anechoic area depicting cervico-vaginal discharge in uterine horns and increased blood flow to uterus (Adams *et al.* 2008).

### **3.2.1.2 Ovarian evaluation**

The examination of ovaries and uterus was carried out at 48 hours interval during an estrous cycle. The data and echogenic structures obtained were saved through images. Further analysis of these ovarian structures such as dominant follicle and corpora lutea was done with ultrasonographic morphometry via inbuilt caliper in ultrasound machine. The diameter (millimeter) of corpus luteum (CL) and dominant follicle (DF) was estimated by taking average of two longest perpendicular dimensions. The diameter of pre-ovulatory follicles was measured and blood supply to larger follicle was evaluated using color Doppler mode. A follicle was categorized as dominant follicle (DF) when its diameter reaches at least 9-10 mm or above. However, the follicles originating from same pool but becoming atretic following dominance were considered subordinate follicles.

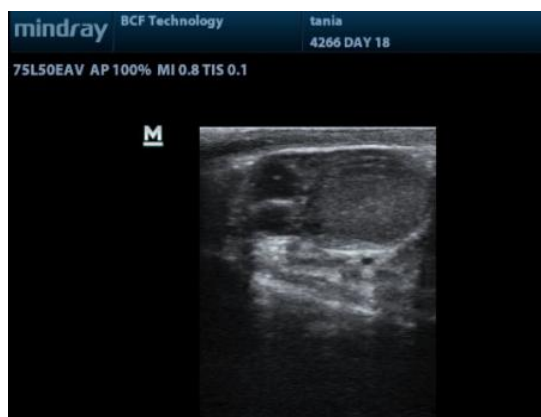


**Plate 3: Sonographic image of pre-ovulatory follicle on the day of estrus (B-mode)**

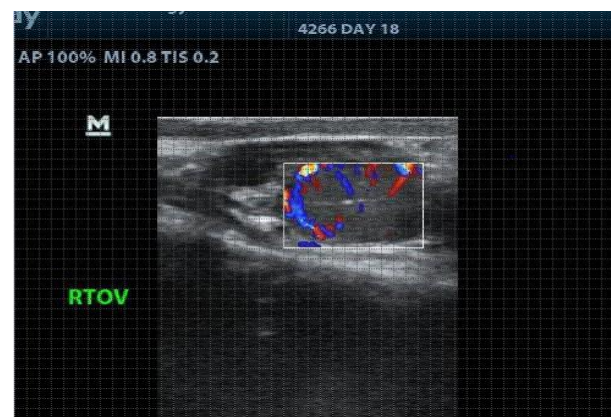
**Plate 4: Sonographic image of CL haemorrhagicum and its vascularity after 48 hours of detected estrus (Color Doppler mode)**

### 3.2.2 Ultrasonographic evaluation of ovulation

Ovulation was characterized by disappearance of largest follicle (anechoic structures) by a hypo-echoic structure (corpus haemorrhagicum; Plate 5) with plenty of blood supply as seen by color Doppler mode (Miura *et al.* 2015). The diameter (millimeter) of CL was calculated every 48 hours after the end of estrus using two larger perpendicular dimensions until the completion of active estrous cycle. To measure the blood flow to CL, it was ensured that entire corpus luteum (CL) falls within the Doppler gate. Vascular perfusion to corpus luteum was analyzed on the cross-sectional image covering the maximum area of the CL using grids (Plate 6). Vascularity of CL (%) = Number of cubes with color Doppler signal on CL / number of cubes covering total CL  $\times$  100.



**Plate 5: B-mode scan of corpus luteum**



**Plate 6: Calculation of vascular perfusion index of corpus luteum using grid analysis**

### 3.2.3 Color Doppler ultrasonography of middle uterine artery (MUA)

Trans-rectal Doppler mode ultrasonography was performed on both the middle uterine arteries for evaluation of uterine blood flow through Doppler indices (Bollwein *et al.* 2000). Doppler indices of significant importance were Resistivity index (RI), Pulsatility index (PI), Time averaged mean velocity (TAMEAN), Time averaged maximum velocity

(TAMAX), Mean blood flow volume (BFV-TAMEAN), Maximum blood flow volume (BFV-TAMAX) and Systole diastole ratio (Sprague *et al.* 2009; Hawkes *et al.* 2016).

A standardized preset was used throughout the examination to minimize alterations. Integration angle between the Doppler ultrasound beam and the flow direction was kept between 30-60° with a color gain of nearly 70. To measure the velocity of wave forms, spectral Doppler mode was used (Plate 7).

Middle uterine artery is a movable vessel (both sides) found within the mesometrium and visualized near the origin from umbilical artery with the color Doppler ultrasonography (Hassan *et al.* 2017). When ultrasound transducer was placed transversely, external iliac artery was easily found at the branching level of aorta. Following external iliac artery caudally, internal iliac artery was identified. About 4cm caudally to internal iliac artery, a common stem umbilical artery and middle uterine artery was found. The blood supply to uterus is mainly from uterine artery and in nulliparous and non-pregnant cows; it is reported to be 2.8 to 5.0 mm in diameter (Ando *et al.* 2004; Hassan *et al.* 2017).

Spectral Doppler ultrasonography was carried out at 5.7 MHz using linear array transducer. Once the uterine artery was confirmed through color Doppler ultrasonography, Doppler gate was placed on the cross-sectional area of the vessel and the spectral Doppler mode was turned on. Doppler gate was adjusted according to size of the vessel. All values regarding uterine blood flow were automatically calculated by inbuilt software of the machine. Values for 3 consecutive wave forms were calculated and average of three calculations of each side were taken at every examination. Assessment of uterine blood flow was done using following parameters; viz. Pulsatility index (PI), Resistivity index (RI), Time averaged mean velocity (TAMEAN), Time averaged maximum velocity (TAMAX), Mean blood flow volume (BFV-TAMEAN), Maximum blood flow volume (BFV-TAMAX) and Systolic diastolic ratio.



**Plate 7: Spectral Doppler image of middle uterine artery**

### 3.2.3.1 Assessment of Doppler indices

#### 3.2.3.1.1 Resistance index (RI)

It was measured as ratio of difference between peak systolic and end diastolic velocity to peak systolic velocity. The RI tends to increase if there is an increase in constriction of distal vascular bed whereas decreased RI values indicate less impedance to blood flow in the vasculature. It is expressed as:

$$RI = (S-D)/S$$

Where S is peak systolic velocity and D is end diastolic velocity.

#### 3.2.3.1.2 Pulsatility index (PI)

PI is also known as mean pulsatility index and often expressed as ratio of difference between peak systolic and end diastolic velocity to average peak systolic velocity. It is expressed as:

$$PI = (S-D)/\text{Velocity}_m$$

Where S is peak systolic velocity, D is end diastolic velocity and  $\text{velocity}_m$  is average velocity of 3 to 4 cardiac cycles.

#### 3.2.3.1.3 Velocity indices (TAMEAN/TAMAX)

TAMEAN/TAMAX was determined by measuring the time-averaged mean/maximum frequency shift over the cardiac cycle. It can be expressed as:

$$TAMEAN = (\text{TAMeF} \times c) / (2F \times \cos \alpha)$$

TAMeF is the time-averaged mean frequency shift over the cardiac cycle, c is ultrasound propagation speed, and F is the transmitted wave frequency and  $\alpha$  is angle between the ultrasonic beam and the blood flow direction.

$$TAMAX = (\text{TAMF} \times c) / (2F \times \cos \alpha)$$

TAMF is the timed-average frequency shift over the cardiac cycle,  $c$  is ultrasound propagation speed, and  $F$  is the transmitted wave frequency and  $\alpha$  is angle between the ultrasonic beam and the blood flow direction (Bollwein *et al.* 2002).

### 3.2.3.1.4 Blood flow volume (BFV)

Mean BFV and maximum BFV was calculated using the diameter of the uterine artery and the mean and maximum blood flow velocity.

$$\text{BFV-TAMEAN [ml/min]} = \text{TAMEAN [cm/s]} \times \pi \times (\text{D [cm]} \times 0.5)^2 \times 60$$

With Mean BFV= mean blood flow volume [ml/min], where the D=sum of the diameters of the right and left uterine arteries [cm], TAMEAN=mean blood flow velocity [cm/s].

$$\text{BFV-TAMAX [ml/min]} = \text{TAMAX [cm/s]} \times \pi \times (\text{D [cm]} \times 0.5)^2 \times 60$$

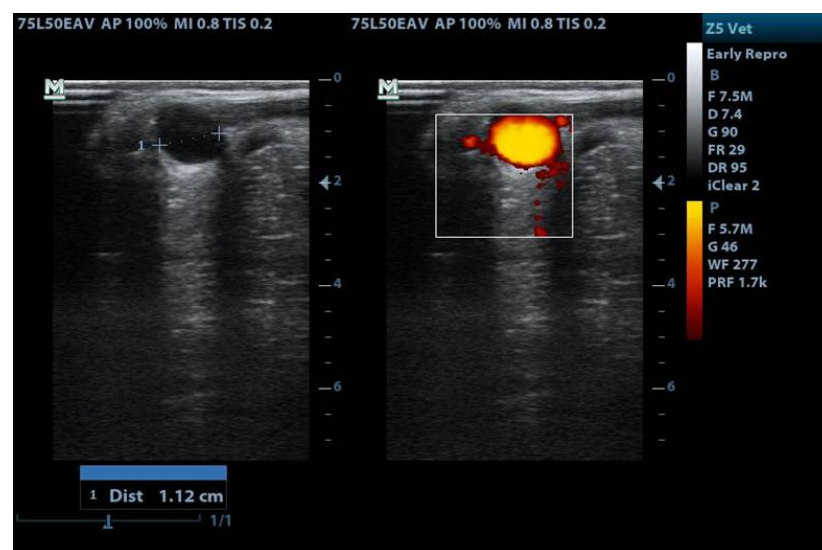
With Maximum BFV=maximum blood flow volume [ml/min], where the D=sum of the diameters of the right and left uterine arteries [cm], TAMAX=maximum blood flow velocity [cm/s] (Bollwein *et al.* 2002).

### 3.2.3.1.5 Systolic diastolic ratio

It is expressed as S/D, where S is peak systolic velocity and D is end diastolic velocity. It is simplest of all indices and errors in the ratio tend to increase with decrease in diastolic velocity.

### 3.2.3.1.6 Cross-sectional area of the middle uterine artery

Diameter of middle uterine artery was calculated by taking mean of three measurements of the diameter made from frozen two-dimensional grey scale images (Plate 8). The cross-sectional area was calculated as  $=\pi \times (\text{D [cm]} \times 0.5)^2$ .



**Plate 8: Cross sectional image of middle uterine artery (B-mode and Power Doppler mode on the left and right side, respectively)**

### **3.3 Statistical analysis**

The recorded data was statistically analyzed using one-way ANOVA, Student's T-Test and linear correlation analysis with NCSS 2020, USA (Version 20.0.1).

## Chapter 4

# *Results and Discussion*

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The advancement of image technologies has contributed significantly in the field of bovine reproduction. Use of real time non-invasive technique to scan tissues and organs, offers a tremendous significance in knowing the physiological mechanisms and diagnosis of pathological conditions (Herzog and Bollwein 2007). Ultrasonography in bovine reproduction has played extensive role in compilation of data regarding ovarian follicular dynamics and subsequent development of several protocols to control ovarian function for assisted reproductive technologies (ARTs) such as timed artificial insemination, superovulation and ovum pick-up (Adams *et al.* 2008). Based on ultrasonographic analysis, most animals' exhibit two or three follicular waves but four wave estrous cycles were also studied (Jaiswal *et al.* 2009). On the other hand, Doppler sonography allows the estimation of blood flow and, can be used to indirectly make inference about the functionality of corpus luteum, follicles and their ability to secrete steroid hormones (Rodgers and Irving-Rodgers 2010). In cattle and other domestic animals, a functional CL is prerequisite for establishment of pregnancy, because progesterone supports preimplantation embryonic development further allowing maternal recognition of pregnancy (Mann and Lamming 1999). Based on these facts, the present research was carried out to study the follicular dynamics and blood flow to ovarian structures via application of trans-rectal ultrasonography (TRUS) during estrous cycle in dairy cows.

### **4.1 Number and growth patterns of follicles within the follicular wave**

Out of 10 cows, 7 exhibited 2-wave estrous cycle, whereas, 3 exhibited 3 wave estrous cycle, in which various parameters such as day of wave onset, maximum diameter of dominant follicle (mm), day of maximum diameter of largest follicle, length of growth phase, duration of dominance, maximum diameter of largest subordinate follicle (mm), inter-wave duration and inter-ovulatory interval were studied.

#### **4.1.1 Follicular dynamics in dairy cows with two- and three-follicular waves**

Follicular dynamics parameters in dairy cows with two- and three-follicular waves during estrous cycle have been shown in Table 1.

**Table 1: Follicular dynamics parameters during estrous cycle in dairy cows (N=10) with two- and three-follicular waves (Mean±S.E.)**

Parameters	Two-wave estrous cycle (n=7)		Three-wave estrous cycle (n=3)		
	First wave	Second wave	First wave	Second wave	Third Wave
Day of wave onset	0.43±0.20	10.28±0.36	0.66±0.33	9.30±0.33	15.33±0.33
Maximum diameter of dominant follicle (mm)	12.46±0.59	13.44±0.56	10.70±0.51 <sup>x</sup>	8.88±0.16 <sup>y</sup>	11.13±0.69 <sup>x</sup>
Day of maximum diameter of dominant follicle	8.57±0.37	17.14±0.26	8.00±0.58	15.67±0.33	20.67±0.67
Length of growth phase (days)	8.80±0.60	9.60±1.21	6.95±0.40	7.78±0.79	8.06±0.56
Duration of dominance (days)	10.80±1.12	8.50±1.16	9.21±0.60	8.28±0.90	8.76±0.72
Maximum diameter of sub-ordinate follicle (mm)	7.24±0.82	7.32±0.50	6.72±0.32	6.86±0.27	7.38±0.30
Inter-wave duration (days)	9.85±0.16		8.64±0.33 (Between 1 <sup>st</sup> and 2 <sup>nd</sup> wave) 6.03±0.33 (Between 2 <sup>nd</sup> and 3 <sup>rd</sup> wave)		
Inter-ovulatory interval (days)	17±0.31 <sup>y</sup>		20.67±0.67 <sup>x</sup>		

<sup>x,y</sup> Values with different superscripts within the same row are significantly different (p<0.05)

Out of 10 cows, seven cows (70%) had two follicular waves, whereas, three cows (30%) had three follicular waves in one estrous cycle (Table 1). Mean estrous cycle length in two-wave and three-wave estrous cycle was 17±0.31 and 20.67±0.67 days, respectively. In two-wave estrous cycle, first wave emerged on day 0.43±0.20 with maximum diameter of dominant follicle (DF) reached 12.46±0.59 mm on day 8.57±0.37. Emergence of second wave occurred on day 10.28±0.36 having DF's maximum diameter of 13.44±0.56 at 17.14±0.26 days. However, Atker *et al.* (2010) recorded the first and second wave emergence on day 3.3 and 12.3 of estrous cycle which is in contrast to findings of present study.

In three-wave estrous cycle, the first wave emerged on day 0.66±0.33 with dominant follicle reaching its maximum size on day 8.00±0.58. Following the first wave, the second and third wave emerged on day 9.30±0.33 and 15.33±0.33 with dominant

follicle reaching maximum diameter on day  $15.67 \pm 0.33$  and  $20.67 \pm 0.67$ , respectively. Present findings were in concurrence with Nosier(2003) who recorded the emergence of first, second and third wave on day 0, 8.7 and 15 of estrous cycle. In synchronicity with findings of current study, Viana *et al.* (2000) also reported that three-wave cyclic pattern had its first wave emergence on day  $0.78 \pm 0.44$  followed by the second and third wave on day  $7.11 \pm 1.05$  and  $13.22 \pm 2.44$ , respectively. As far as size of dominant follicle was concerned, second wave had a significantly smaller size ( $p < 0.05$ ) as compared to first and third wave.

In two-wave estrous cycle, inter-wave duration was  $9.85 \pm 0.16$  days, whereas, it was  $8.64 \pm 0.33$  and  $6.03 \pm 0.33$  days between first and second, second and third wave, respectively, in three-wave estrous cycle. Present study also revealed that the inter-ovulatory interval in two- and three follicular-wave estrous cycles was  $17 \pm 0.31$  and  $20.67 \pm 0.67$  days, respectively, and therefore, the length of inter-ovulatory interval in two- wave estrous cycle was significantly shorter ( $p < 0.05$ ) than three-wave estrous cycle. Patel *et al.* (2006) reported significantly longer estrous cycle length for three-wave estrous cycle as observed for the present study. However, Hadiya *et al.* (2016) recorded no statistical difference in length and duration of estrous cycle between two- and three-wave cycles. A higher incidence of cycle with three-follicular waves, although was observed by many authors in European (Sirois and Fortune 1988; Savio *et al.* 1988) and Zebu cows (Gambini *et al.* 1998) which is in contrary to the present findings.

#### 4.1.2 Pattern of follicular development on ovaries in two- and three-wave estrous cycle

Ovarian follicle diameter during estrous cycle in dairy cows with two- and three-follicular waves has been shown in Table 2.

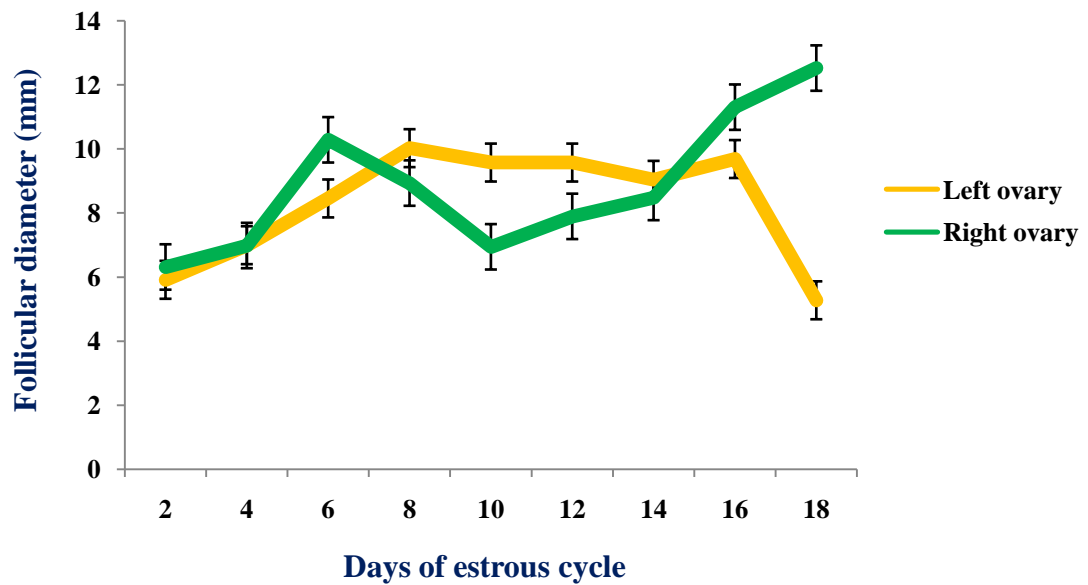
**Table 2: Ovarian follicle diameter (mm) during an estrous cycle in dairy cows (N=10) with two- and three- follicular waves (Mean±S.E.)**

Day of TRUS	Mean follicular diameter (mm)			
	Two-wave estrous cycle (n=7)		Three-wave estrous cycle (n=3)	
	Left Ovary	Right Ovary	Left Ovary	Right Ovary
Day 2	$5.91 \pm 0.66$ (4.05-7.80)	$6.31 \pm 0.45$ (4.80-7.90)	$5.60 \pm 0.45$ (4.75-6.30)	$6.50 \pm 0.41$ (5.80-7.25)
Day 4	$6.99 \pm 0.72$ (5.10-9.15)	$6.98 \pm 1.01$ (5.32-10.23)	$6.23 \pm 0.47$ (5.70-6.70)	$6.20 \pm 1.00$ (5.20-7.20)

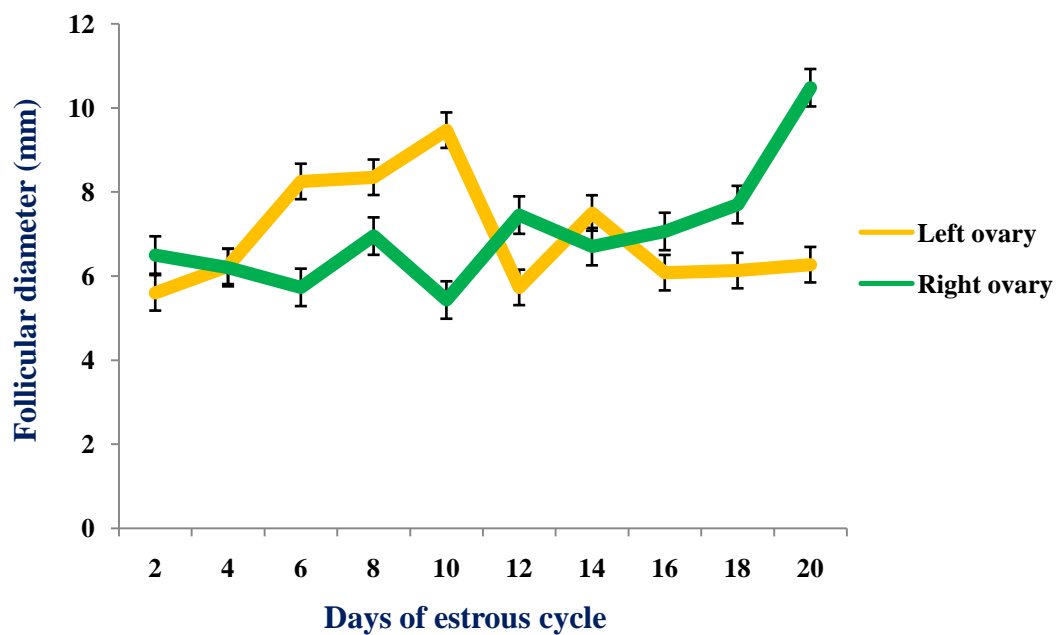
<b>Day 6</b>	8.45±1.12 (5.10-10.60)	10.28±1.34 (7.35-13.50)	8.25±1.75 (6.50-10.00)	5.73±1.48 (4.20-7.20)
<b>Day 8</b>	10.02±1.49 (6.50-13.75)	8.93±1.93 (5.90-14.50)	8.35±0.72 (4.80-11.70)	6.95±3.45 (3.50-10.40)
<b>Day 10</b>	9.57±1.41 (5.24-13.05)	6.94±1.49 (3.50-11.35)	9.47±0.47 (9.00-9.95)	5.43±0.83 (4.60-6.25)
<b>Day 12</b>	9.57±1.16 (5.25-13.10)	7.89±1.70 (4.50-10.50)	5.73±0.83 (4.90-6.56)	7.45±1.65 (5.80-9.10)
<b>Day 14</b>	9.03±0.78 (5.70-12.20)	8.48±1.00 (4.60-12.10)	7.50±0.63 (6.70-7.10)	6.70±0.78 (5.80-8.20)
<b>Day 16</b>	9.68±1.23 (6.20-12.50)	11.30±2.16 (5.70-16.15)	6.08±1.56 (4.35-9.20)	7.06±0.82 (6.05-8.70)
<b>Day 18</b>	5.27±1.07 (4.20-6.35)	12.52±0.63 (11.90-13.15)	6.13±0.67 (5.40-6.80)	7.70±0.20 (7.50-7.90)
<b>Day 20</b>	-	-	6.27±0.89 (5.05-8.00)	10.48±1.09 (8.75-12.50)

During present study, two- and three-wave estrous cycle had the left ovary more active initially but with the progression of estrous cycle into the luteal phase, right ovary became more active in terms of follicular activity (Table 2; Figure 1 and 2). Thus, frequency of dominant follicles (ovulatory) in right ovary was much higher as compared to left ovary which was in complete agreement with Muraya (2013) who also reported the same findings. In present study, 70 per cent cows had ovulations occurring from right ovary which was in agreement with the findings of Stevenson (2019) where 60-65 per cent ovulations were from right ovary. In concurrence with present findings, Kidder *et al.* (1952) reported 56.50 and 43.50 per cent ovulations from right and left ovary, respectively, which may be due to proximity of left ovary to rumen and other extrinsic factors such as temperature or pressure fluctuations and mechanical contractions of the rumen and further, leads to greater developmental potential of oocytes originating from right ovaries (Karamishabankareh *et al.* 2015). A characteristic pattern of follicular waves, with the initial development of a group of 3 to 5 mm follicles followed by selection, dominance and atresia of dominant follicle was observed (Figure 1 and 2).

**Figure 1: Ovarian follicle diameter (mm) during an estrous cycle in dairy cows with two-follicular waves**



**Figure 2: Ovarian follicle diameter (mm) during an estrous cycle in dairy cows with three-follicular waves**



## 4.2 Dynamics of luteal development and vascularity

### 4.2.1 Corpus luteum diameter (mm) and vascularity (%) during different luteal phases

Corpus luteum (CL) diameter (mm) and vascularity (%) during different luteal phases in estrous cycle with two and three follicular waves is shown in Table 3.

**Table 3: Corpus luteum diameter (mm) and vascularity (%) during different luteal phases of an estrous cycle in dairy cows (N=10) with two- and three-follicular waves and their pregnancy status (Mean±S.E.)**

Parameters	Two-wave estrous cycle (n=7)			Three-wave estrous cycle (n=3)		
	Early luteal phase (Day 1-3)	Mid luteal phase (Day 4-10)	Late luteal phase (Day 11-18)	Early luteal phase (Day 1-4)	Mid luteal phase (Day 5-12)	Late luteal phase (Day 13-22)
<b>Corpus luteum diameter (mm)</b>	13.30±0.44 <sup>b</sup> (11.85-14.60)	20.85±0.95 <sup>a</sup> (11.9-27.9)	18.32±0.85 <sup>a</sup> (13.1-24.3)	11.75±1.27 <sup>b</sup> (9.35-14.35)	19.29±0.58 <sup>a</sup> (18.05-22.6)	19.13±0.47 <sup>a</sup> (16.68-21.15)
<b>Corpus luteum vascularity (%)</b>	21.26±1.46 <sup>b</sup> (18.35-26.41)	38.31±1.46 <sup>a</sup> (28.12-48.42)	24.79±3.15 <sup>bQ</sup> (2.89-41.75)	24.34±4.17 <sup>y</sup> (15.45-32.3)	39.12±1.88 <sup>x</sup> (32.13-45.52)	39.17±1.31 <sup>xP</sup> (31.3-44.4)
<b>Pregnancy status (%)</b>	0/7 (0)			3/3 (100)		

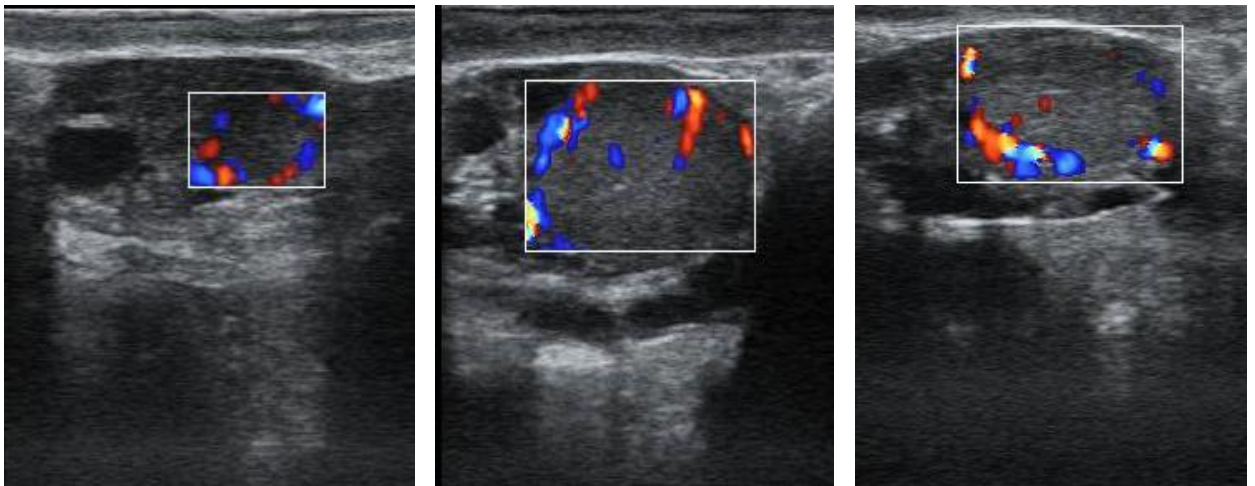
<sup>a,b</sup>Values with different superscripts within the same row for the same wave and parameter are significantly different (p<0.01)

<sup>x,y</sup>Values with different superscripts within the same row for the same wave and parameter are significantly different (p<0.05)

<sup>P,Q</sup>Values with different superscripts within the same row for the same luteal phase and parameter are significantly different (p<0.05)

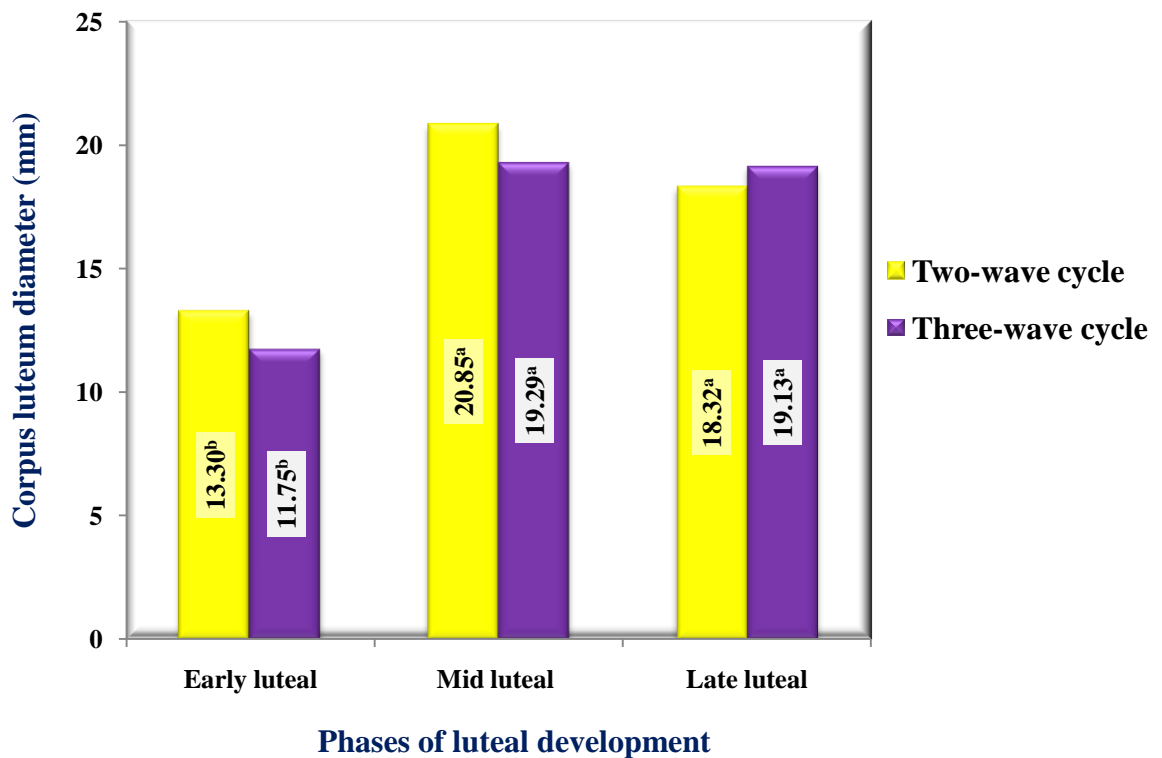
Relationship of CL diameter and vascularity in relation to plasma progesterone secretion has been evaluated in cows using color Doppler ultrasonography (Pinaffi *et al.* 2015; de Tarso *et al.* 2017). In present study, luteal phase has been divided into three phases i.e. early, mid and late luteal phase (Plate 9). In two-wave estrous cycle, mean corpus luteum diameter during early luteal phase was  $13.30 \pm 0.44$  mm, showed significant increase ( $p < 0.01$ ) in diameter i.e.  $20.85 \pm 0.95$  mm, during mid-luteal phase before getting decreased non-significantly ( $p < 0.05$ ) to  $18.32 \pm 0.85$  mm during late luteal phase, thus, indicating regression of corpus luteum.

In three-wave estrous cycle, mean corpus luteum diameter during early luteal phase was  $11.75 \pm 1.27$  mm, increased to  $19.29 \pm 0.58$  mm in mid luteal phase and remained almost same i.e.  $19.13 \pm 0.47$  mm, in late luteal phase. The significant difference ( $p < 0.01$ ) was recorded for size of CL between mid to late and early luteal phase which corresponds to the higher rate of growth during the later phases of estrous cycle. In concurrence with present findings, Gaur and Purohit (2019) reported a precipitous fall in blood flow to the CL in non-pregnant buffaloes from Day 15-21 of the estrous cycle and thus, represents a quick and reliable diagnostic test for the early pregnancy diagnosis (Siqueira *et al.* 2013). Similarly, the changes in CL diameter and vascularity were also concomitant to plasma progesterone concentration in other studies (de Tarso *et al.* 2017).



**Plate 9: Corpus Luteum blood flow during early luteal phase (day 2-4), mid luteal phase (day 5-12), and late luteal phase (day 13-22)**

**Figure 3: Corpus luteum diameter (mm) during different luteal phases of an estrous cycle in dairy cows with two- and three-follicular waves**

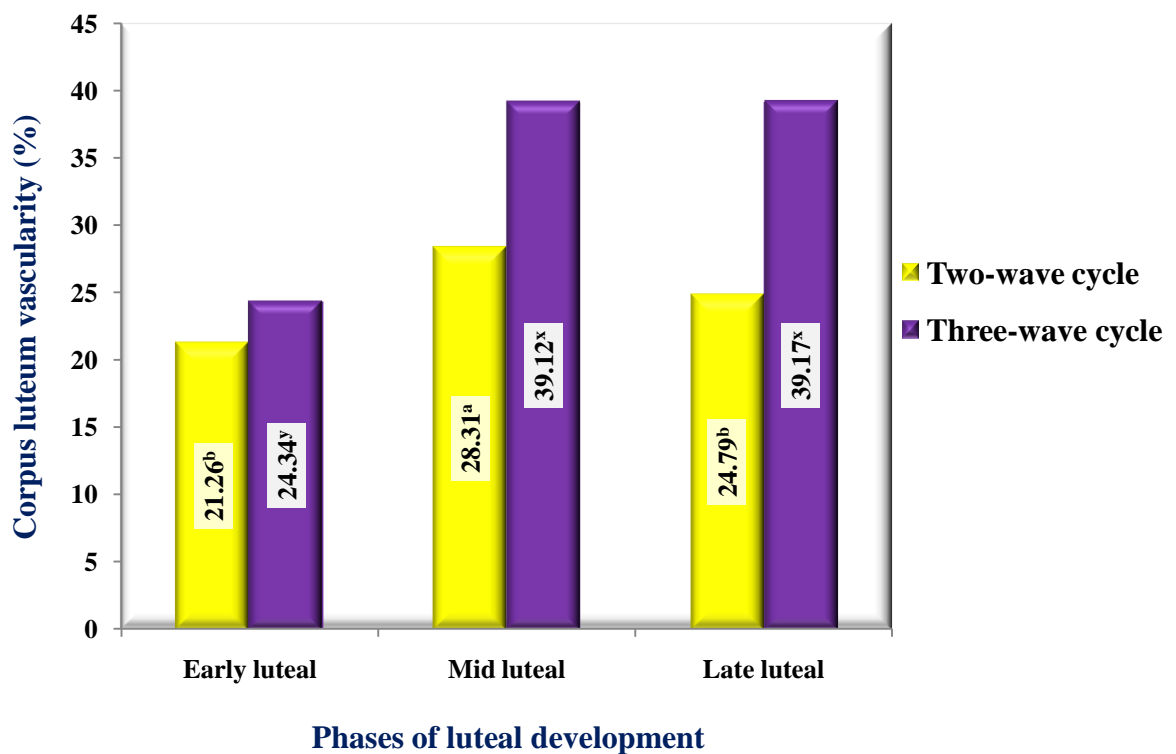


Growth of the CL and its functions are dependent on blood supply (Acosta and Miyamoto 2004). Following ovulation, CL size and vascularity increase but no greater increase is seen during early luteal phase as it occurs during mid-luteal phase due to angiogenic growth factors such as Fibroblast Growth Factor and Vascular Endothelial Growth Factor (Luttgenau *et al.* 2011). Vascularity of corpus luteum in two-wave estrous cycle was  $21.26 \pm 1.46$ , increased significantly ( $p < 0.01$ ) to  $38.31 \pm 1.46$  before decreasing significantly ( $p < 0.01$ ) to  $24.79 \pm 3.15$  per cent during early, mid and late luteal phase, respectively. In three-wave estrous cycle, CL vascularity during early luteal phase was low i.e.  $24.34 \pm 4.17$  per cent, and increased significantly ( $p < 0.05$ ) to  $39.12 \pm 1.88$  per cent in mid luteal phase and remained almost same in late luteal phase i.e.  $39.17 \pm 1.31$  per cent.

Acknowledging the findings of present study, the blood supply to the CL increases in parallel with its growth, followed by an acute increase in blood flow to the mature CL prior to luteal regression (Matsui and Miyamoto 2009). Herzog and Bollwein (2007) studied a positive correlation between plasma progesterone concentrations and CL blood flow throughout the estrous cycle, reflecting the development and regression of luteal tissue. Herzog *et al.* (2010) concluded that correlations between progesterone and CL size are not as strong as, between Corpus luteum blood flow (CLBF) and plasma progesterone,

reflecting that Doppler sonography improves the accuracy of diagnosis by providing the physiological information of the organ. However, the CL vascularity is initially visualized on its boundary because color Doppler sonography is very much unable to differentiate between blood vessels in the center because of their low blood flow velocity (Luttgenau and Bollwein 2014).

**Figure 4: Corpus luteum vascularity (%) during different luteal phases of an estrous cycle in dairy cows with two- and three-follicular waves**



#### 4.2.2 Corpus luteum diameter, vascularity and their correlation irrespective of follicular waves

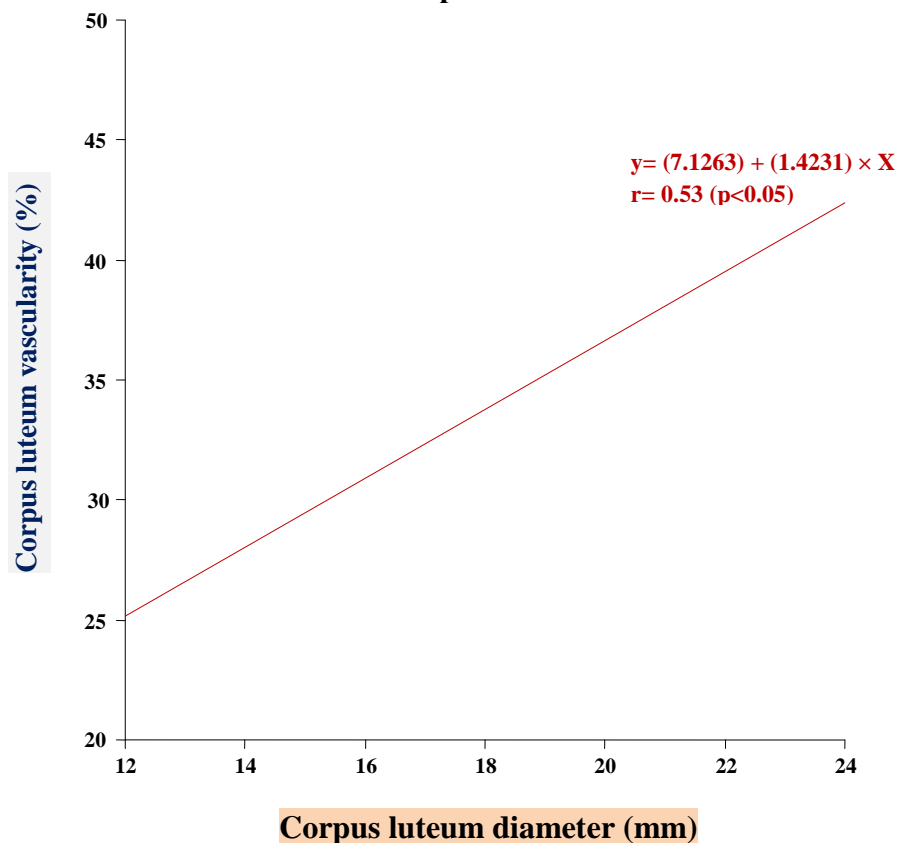
Development of corpus luteum (mm), change in vascularity (%) and inter-correlation between these two parameters irrespective of number of follicular waves is depicted in Table 2 and Figure 5.

**Table 4: Corpus luteum diameter (mm) and vascularity (%) during estrous cycle in dairy cows (N=10) irrespective of follicular waves and different luteal phases (Mean±S.E.)**

Day of TRUS	Corpus luteum diameter (mm)	Corpus luteum vascularity (%)
Day 2	12.83±0.59 (9.80-14.60)	20.11±1.31 (15.45-26.41)

<b>Day 4</b>	14.54±1.82 (9.35-19.00)	32.74±2.56 (30.55-40.00)
<b>Day 6</b>	19.97±0.97 (17.40-22.45)	38.96±1.54 (32.46-43.52)
<b>Day 8</b>	19.14±1.25 (15.85-23.60)	37.17±5.64 (30.80-48.42)
<b>Day 10</b>	20.94±0.77 (18.50-24.63)	39.27±1.62 (32.13-45.5)
<b>Day 12</b>	22.07±0.80 (19.50-24.30)	37.46±1.92 (33.19-45.52)
<b>Day 14</b>	20.65±0.75 (16.34-23.25)	32.16±2.74 (22.60-42.42)
<b>Day 16</b>	18.39±1.23 (13.10-21.15)	20.95±5.46 (12.80-34.90)
<b>Day 18</b>	17.87±1.63 (16.25-19.50)	42.30±2.10 (40.20-44.40)
<b>Day 20</b>	18.10±0.59 (7.20-19.20; n=3)	36.95±4.25 (32.70-41.20; n=3)
<b>Day 22</b>	17.93±1.27 (16.65-19.20; n=3)	39.75±1.05 (38.70-40.80; n=3)

**Figure 5: Linear correlation and regression between corpus luteum diameter (mm) and vascularity (%) during an estrous cycle in dairy cows (N=10) irrespective of follicular waves' and different luteal phases**



Until several years ago, morphological and functional changes in the CL during the estrous cycle are monitored by determining plasma  $P_4$  concentrations (Ribadu *et al.* 1994). However, new technique Doppler sonography has been used to examine the blood circulation of the CL in humans (Bourne *et al.* 1996). In present study, during development of the CL in the first week after ovulation, the size and vascularity increased rapidly from day 2 to 4, however, the size and vascularity of CL increases to attain peak around day 12 i.e. in the mid-luteal phase (day 5 to 12). During this phase, most steroidogenic cells are adjacent to one or more capillaries and CL has the highest blood flow (Reynolds *et al.* 2000). Adequate blood flow to the CL is also essential for the release of progesterone into the circulation (Acosta *et al.* 2002). Although decline in luteal size and blood flow was seen after day 14 in the present study which is mainly due to failure of recognition of maternal pregnancy signals and secretion of luteolytic hormone i.e. prostaglandin  $F_{2\alpha}$ , from uterine endometrium and resulting in regression of the CL (Schams and Berisha 2004; Ginther *et al.* 2009).

The significant positive linear correlation ( $r=0.53$ ;  $p<0.05$ ) was found between corpus luteum diameter (mm) and vascularity (%) in dairy cows (Figure 5). Regression equation analysis revealed that a single unit increase in diameter of CL resulted in 1.42 times increase in CL vascularity during different luteal phases of estrous cycle. Following ovulation, CL is one of the highly vascularized organs and receives ample amount of blood flow per unit of tissue than any other organ in the body (Wiltbank *et al.* 1988). During its initial development (48–120 h), a gradual increase in the blood flow around the CL occurs simultaneously with an increase in its volume and serum progesterone concentration (Acosta *et al.* 2003), which indicates that local blood flow in the CL is related to its potential to produce and release progesterone. As the blood delivers steroid precursors and gonadotropins (Janson *et al.* 1981) and most of the steroidogenic cells are in close contact with the capillaries (Reynolds *et al.* 1992), the vascularity plays a key role in maintaining the functionality of CL.

#### **4.3 Spectral Doppler haemodynamic indices of middle uterine artery**

Spectral Doppler haemodynamic indices i.e. RI, PI, TAMEAN, TAMAX, BFV-TAMEAN, BFV-TAMAX, systolic diastolic ratio and diameter of middle uterine artery (MUA), during estrous cycle in dairy cows are presented in Table 5 and depicted in Figure 6 to 13.

**Table 5: Doppler haemodynamic indices of middle uterine arteries in dairy cows (N=10) during an estrous cycle (Mean±S.E.)**

Day of estrous cycle	Side of MUA in relation to POF	Resistivity index (RI)	Pulsatility index (PI)	Time averaged mean velocity (cm/sec)	Time averaged maximum velocity (cm/sec)	Diameter of middle uterine artery	Blood flow volume-TAMEAN (mL/min)	Blood flow volume-TAMAX (mL/min)	Systolic diastolic ratio
Day 0	Ipsilateral	0.31±0.08 <sup>b</sup>	0.55±0.14 <sup>b</sup>	31.47±2.20 <sup>a</sup>	55.16±1.36 <sup>a</sup>	12.58±0.68 <sup>x</sup>	2353.58±289.81 <sup>a</sup>	4178.13±490.67 <sup>a</sup>	1.58±0.26 <sup>b</sup>
	Contralateral	0.71±0.09 <sup>a</sup>	1.47±0.14 <sup>a</sup>	20.12±2.02 <sup>b</sup>	39.07±2.55 <sup>b</sup>	10.73±0.36 <sup>y</sup>	1072.96±86.18 <sup>b</sup>	2095.55±123.40 <sup>b</sup>	3.39±0.42 <sup>a</sup>
Day 2	Ipsilateral	0.50±0.12	1.00±0.25	25.26±2.33	50.53±3.35	12.27±0.29 <sup>a</sup>	1902.97±214.24 <sup>x</sup>	3580.28±400.36 <sup>x</sup>	2.54±0.41
	Contralateral	0.60±0.04	1.14±0.10	22.18±2.31	41.17±3.61	10.70±0.37 <sup>b</sup>	1260.36±165.16 <sup>y</sup>	2377.54±236.27 <sup>y</sup>	2.60±0.29
Day 4	Ipsilateral	0.66±0.09	1.36±0.14	23.07±5.94	46.88±9.72	11.37±0.38 <sup>a</sup>	1510.29±408.24	2907.99±477.08	3.12±0.41
	Contralateral	0.59±0.14	1.47±0.30	16.52±3.77	31.95±8.24	9.68±0.46 <sup>b</sup>	736.22±267.09	1452.05±541.38	3.20±0.68
Day 6	Ipsilateral	0.45±0.09	0.74±0.17	23.09±3.23	39.98±4.73	11.34±0.76	1546.52±375.54	2500.15±542.88	2.12±0.37
	Contralateral	0.54±0.06	0.97±0.15	23.59±2.04	42.27±3.28	10.46±0.34	1198.72±185.05	2193.21±333.39	2.36±0.32
Day 8	Ipsilateral	0.59±0.06	1.06±0.16	25.41±5.54	49.05±7.48	11.34±0.31	1473.28±351.58	2744.03±559.29	2.39±0.42
	Contralateral	0.50±0.10	0.99±0.42	17.20±2.52	31.73±4.83	10.35±0.59	990.38±220.74	1766.73±397.51	2.62±0.76
Day 10	Ipsilateral	0.69±0.03 <sup>a</sup>	1.39±0.08 <sup>a</sup>	24.65±5.43	38.86±7.43	10.22±0.29	963.31±96.31	1622.78±102.76	3.34±0.26 <sup>a</sup>
	Contralateral	0.48±0.05 <sup>b</sup>	0.94±0.12 <sup>b</sup>	16.09±2.28	28.87±2.72	10.08±0.49	726.19±132.17	1338.74±140.00	1.84±0.16 <sup>b</sup>
Day 12	Ipsilateral	0.69±0.05	1.21±0.12	25.24±4.06	45.83±6.42	10.82±0.52	1467.17±262.27	2695.42±455.68	3.55±0.54
	Contralateral	0.67±0.02	1.14±0.19	24.52±4.19	36.32±3.13	10.34±0.52	1272.96±267.47	1957.97±305.29	2.93±0.18

**Table 5 cont.**

Table 5 cont.

Day of estrous cycle	Side of MUA in relation to POF	Resistivity index (RI)	Pulsatility index (PI)	Time averaged mean velocity (cm/sec)	Time averaged maximum velocity (cm/sec)	Diameter of middle uterine artery	Blood flow volume-TAMEAN (mL/min)	Blood flow volume-TAMAX (mL/min)	Systolic diastolic ratio
Day 14	Ipsilateral	0.49±0.12	0.94±0.26	19.33±4.76	35.19±7.27	11.48±0.39	1189.49±352.53	2242.39±610.91	3.35±1.52
	Contralateral	0.69±0.03	1.40±0.16	18.74±1.41	34.02±2.30	10.53±0.43	1007.69±102.66	1782.27±213.53	3.39±0.35
Day 16	Ipsilateral	0.62±0.03	1.29±0.84	21.71±2.62	38.43±3.84	11.08±0.50	1284.05±223.02	2272.78±352.74	2.42±0.26
	Contralateral	0.59±0.05	1.27±0.16	21.01±2.89	35.90±3.39	10.91±0.83	1283.34±306.27	2157.32±406.75	2.91±0.44
Day 18	Ipsilateral	0.63±0.13	1.24±0.39	21.98±1.02 <sup>x</sup>	42.57±6.89	12.50±1.00	1614.5±331.51	3183.48±999.47	2.92±0.59
	Contralateral	0.71±0.01	1.59±0.37	15.39±2.96 <sup>y</sup>	30.63±4.99	10.65±0.65	907.67±207.41	1656.07±448.44	3.44±0.05
Day 20	Ipsilateral	0.74±0.01	1.62±0.02	16.92±1.43	28.63±2.66	11.75±0.15	881.02±212.68	1542.35±311.71	3.81±0.12
	Contralateral	0.76±0.05	1.47±0.14	15.39±2.96	29.15±8.22	10.35±0.95	798.08±49.45	1419.95±95.07	4.60±1.10
Day 22	Ipsilateral	0.76±0.02 <sup>a</sup>	1.19±0.47	-	-	10.30±2.20	-	-	3.93±0.52
	Contralateral	0.42±0.03 <sup>b</sup>	1.01±0.58	-	-	9.15±1.05	-	-	2.58±1.40

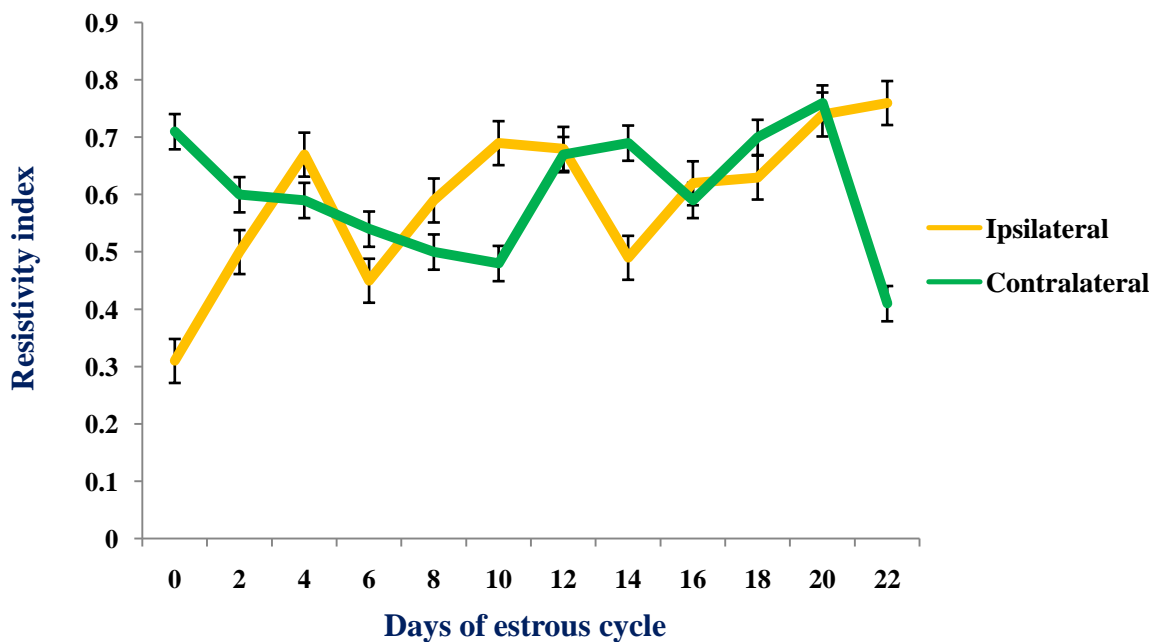
<sup>a,b</sup>Values with different superscripts within the same column for the same parameter and day are significantly different (p<0.01)

<sup>x,y</sup>Values with different superscripts within the same row for the same parameter and day are significantly different (p<0.05)

### 4.3.1 Resistivity index (RI)

In present study, the resistivity index was found to be significantly lower ( $p < 0.01$ ) in ipsilateral artery as compared to contralateral artery on day 0 i.e. day of estrus, due to decreased impedance to blood flow in the ipsilateral artery (Table 5; Figure 6) as a sequel to increased estrogen (vasodilation) levels and thus, resulting in increased blood flow to uterus (Bollwein *et al.* 2000; Rawy *et al.* 2018). Silva (2011) recorded higher value of RI during constriction of distal vascular bed and conversely, a low value of RI during decreased impedance to blood flow in the distal vascular bed on the day of estrus which is in agreement with our findings. Hassan *et al.* (2017) reported a higher RI of uterine arteries during diestrus as compared to estrus and ovulation. RI was also found to be significantly low ( $p < 0.01$ ) on the day of emergence of dominant follicles of anovulatory waves seen during the luteal phase due to secretion of 17- $\beta$  estradiol by dominant follicle (Mattioli *et al.* 2001).

**Figure 6: Change in resistivity index of middle uterine arteries during an estrous cycle in dairy cows**

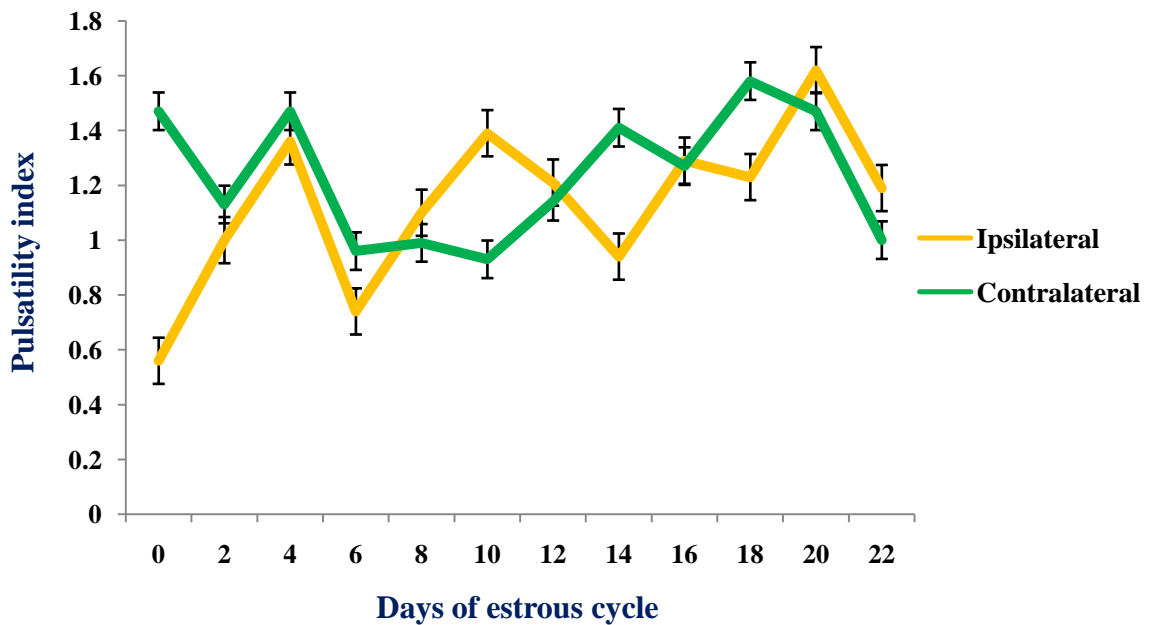


### 4.3.2 Pulsatility index (PI)

Pulsatility index demonstrates the resistance in the vascular bed distal to the site of measurement. PI was recorded to be significantly low ( $p < 0.01$ ) in the ipsilateral artery as compared to contralateral artery on Day 0 i.e. day of estrus, (Table 5; Figure 7) due to increased blood flow on the arterial side ipsilateral to pre-ovulatory follicle (Bollwein *et al.* 2000). Our study was in concurrence with Moonmanee *et al.* (2018), who reported the value of RI and PI were significantly low on ovulatory side uterine artery as compared to

contralateral side. The significant drop ( $p < 0.01$ ) in value of PI was seen on day 10 due to the secretion of  $17\text{-}\beta$  estradiol by dominant follicle and subsequent, increase in vascular perfusion (Hassan *et al.* 2017). Pulsatility index was negatively correlated with the vascular perfusion and thus, the increase of PI in the uterine arteries might be due to vasoconstriction of the blood vessels at the point of measurement (Elmetwally and Meinecke-Tillmann 2012; Elmetwally 2016).

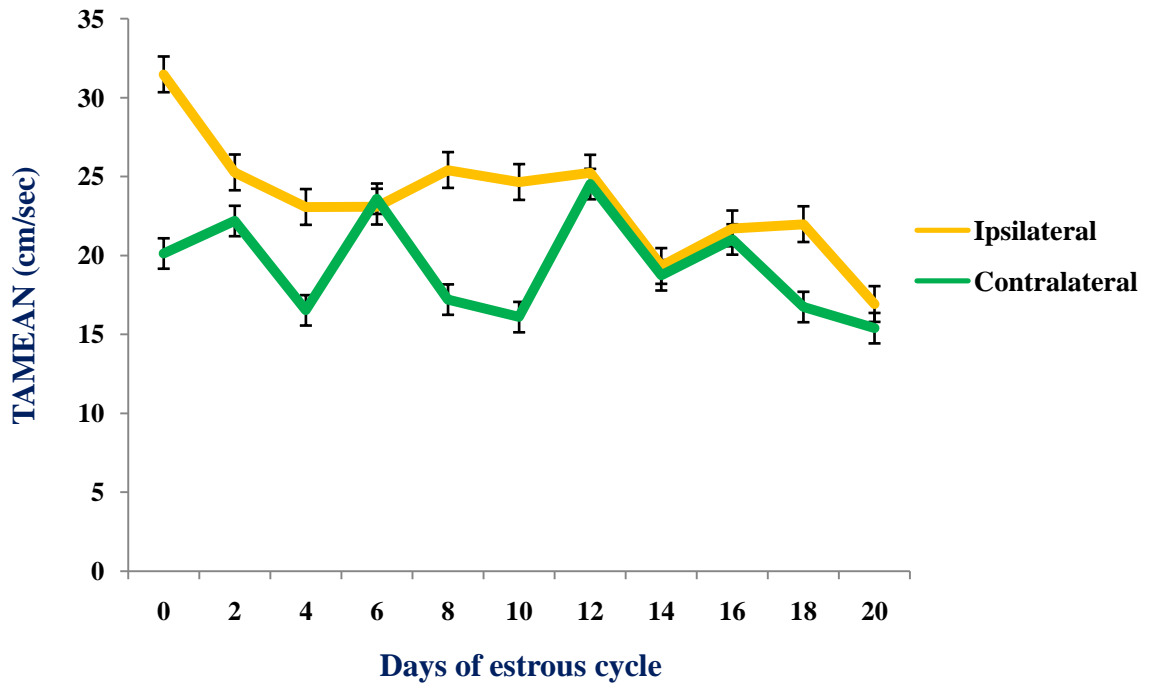
**Figure 7: Change in pulsatility index of middle uterine arteries during an estrous cycle in dairy cows**



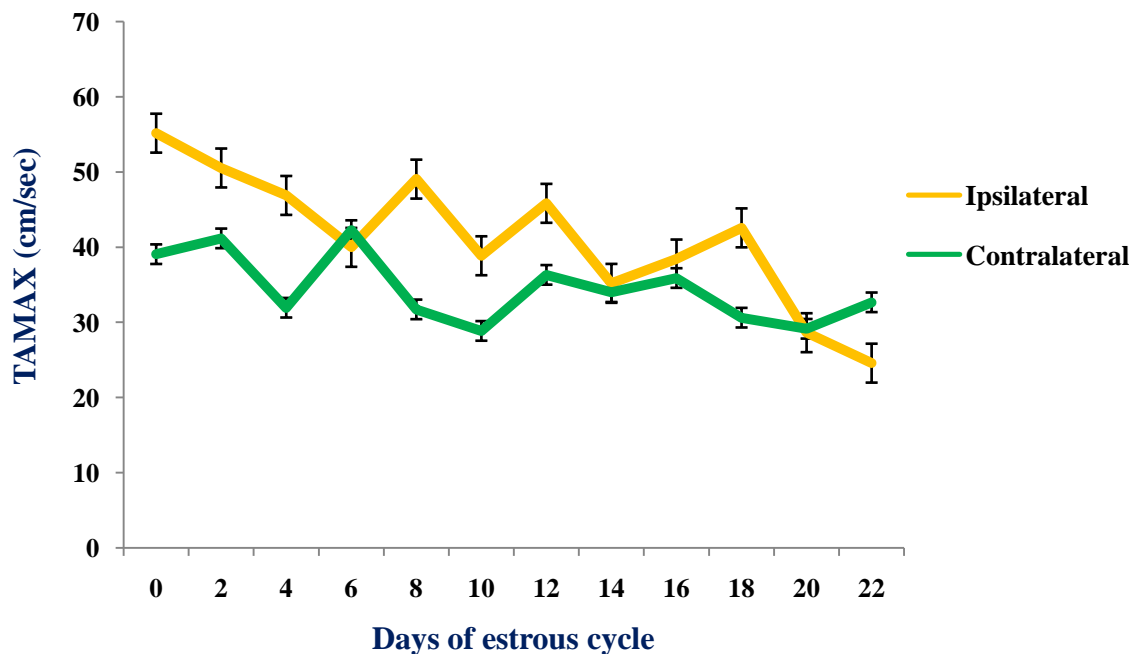
#### 4.3.3 Time averaged mean velocity (TAMEAN) and time averaged maximum velocity (TAMAX)

TAMEAN and TAMAX were significantly higher ( $p < 0.01$ ) in ipsilateral artery as compared to contralateral artery on the day 0 i.e. day of estrus (Table 5; Figure 8 and 9). Our study was in concurrence with another study where a positive correlation between velocity of blood flow and estrogen concentration and negative correlation between RI and plasma estrogen levels as reported by Bollwein *et al.* (2000). Similarly, mean velocity of blood flow during the follicular phase was higher than as compared to luteal phase in both the uterine arteries ipsilateral and contralateral to pre-ovulatory follicle (Ando *et al.* 2004). Debertolis *et al.* (2016) concluded that blood flow to the uterus is controlled mainly by estradiol and progesterone hormone concentrations.

**Figure 8: Change in TAMEAN (cm/sec) of middle uterine arteries during an estrous cycle in dairy cows**



**Figure 9: Change in TAMAX (cm/sec) of middle uterine arteries during an estrous cycle in dairy cows**

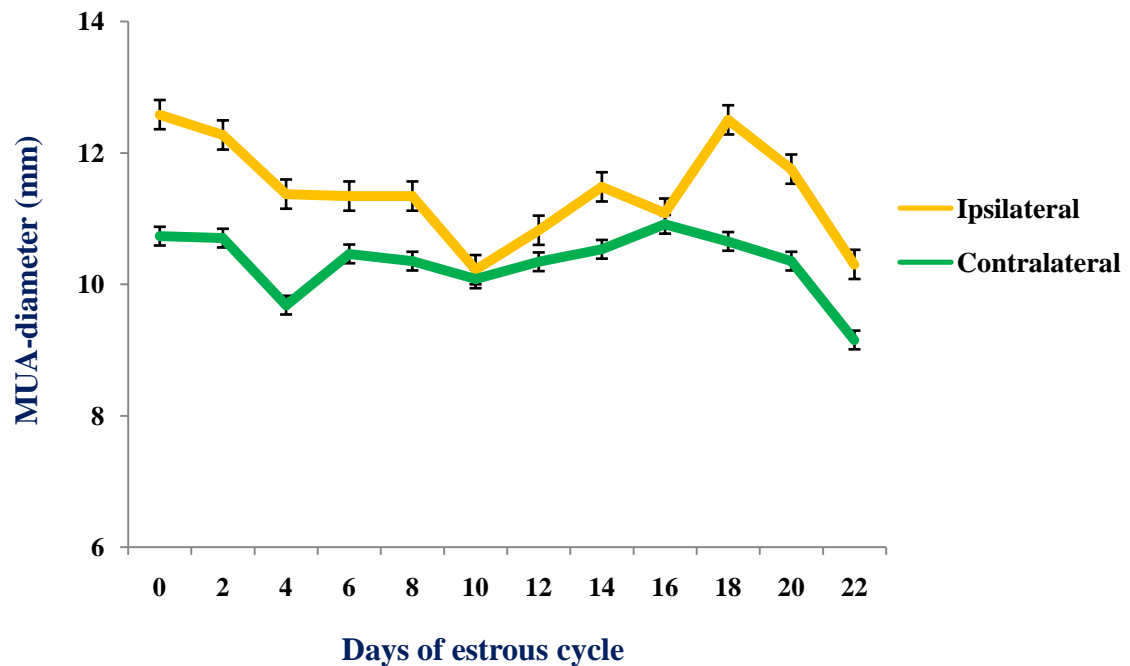


#### 4.3.5 Diameter of middle uterine artery (MUA)

Diameter of MUA was found to be significantly higher ( $p < 0.05$ ) in ipsilateral artery as compared to contralateral artery on the day of estrus (Table 5; Figure 10). The diameter of MUA following day 0 decreased gradually and increased around Day 18 again in response to beginning of next estrous cycle (Bollwein *et al.* 2000). The

findings of present study were in agreement with Bollwein *et al.* (2000); Panarace *et al.* (2006); Pinaffi *et al.* (2017) where increased diameter of middle uterine artery of any side demarcates the side of ovulation. As reported in literature, the diameter of middle uterine artery increases under the influence of potent vasodilator i.e. estrogen, therefore, with decrease in concentration of estrogen, diameter also starts decreasing which happens during the luteal phase (Acosta *et al.* 2003; Chen *et al.* 2004).

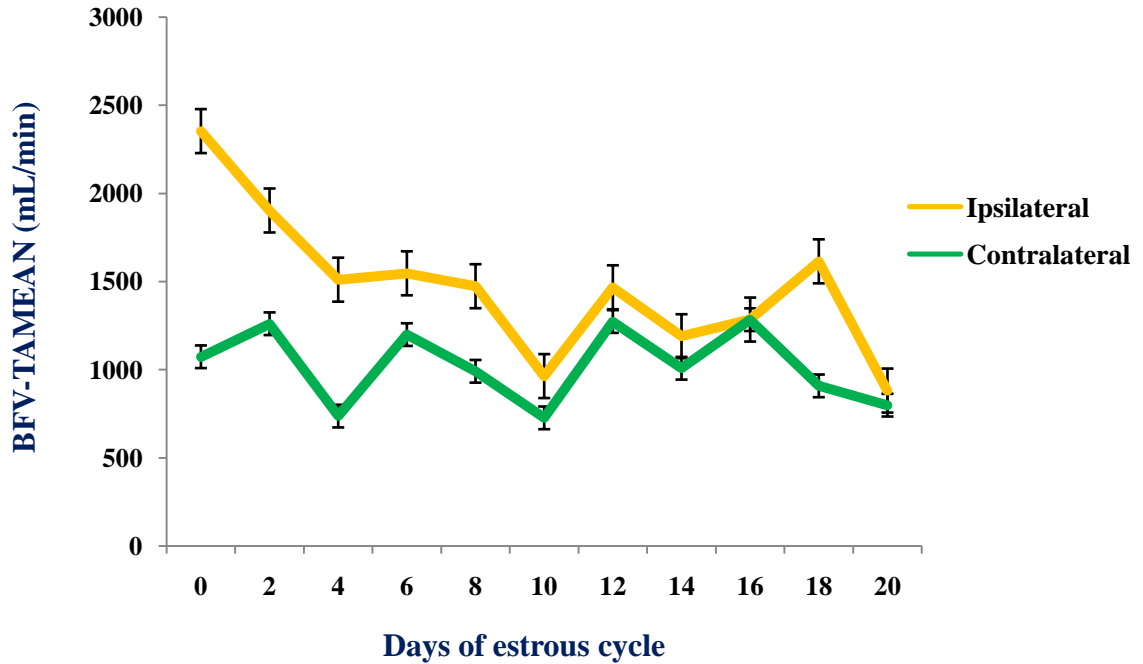
**Figure 10: Change in MUA-diameter (mm) of middle uterine arteries during an estrous cycle in dairy cows**



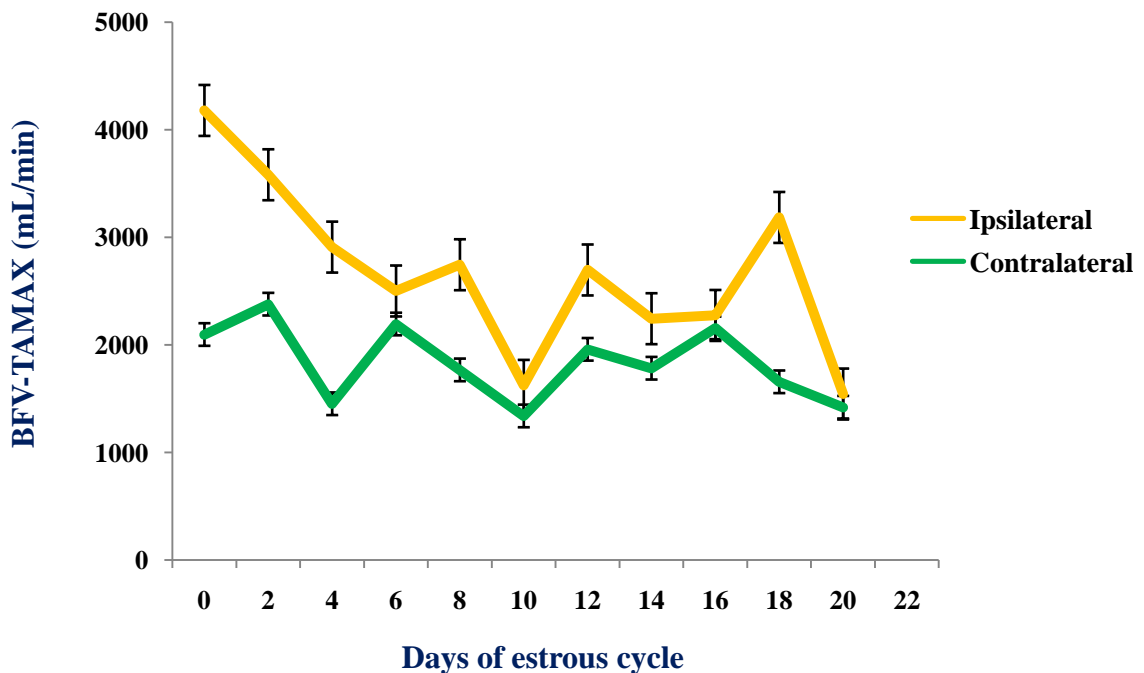
#### 4.3.6 Timed average mean and maximum blood flow volume (BFV-TAMEAN and BFV-TAMAX)

The BFV-TAMEAN and BFV-TAMAX were significantly higher ( $p < 0.01$ ) on day 0 and 2 in ipsilateral as compared to contralateral uterine artery during the estrous cycle (Table 5; Figure 11 and 12) due to increased estrogen concentration and subsequently, resulting in higher blood flow to the uterus (Bollwein *et al.* 2000). Present study was in concurrence with findings of Honnenset *et al.* (2008) where higher blood flow volume, blood flow velocities, estrogen levels, lower PI values and progesterone concentrations at the time of ovulation as compared to non-ovulating cows. Also, uterine blood flow and estrogen induced vasodilation are essential for follicular growth during pre-ovulatory phase in cows (Moonmanee *et al.* 2018).

**Figure 11: Change in BFV-TAMEAN (ml/min) of middle uterine arteries during an estrous cycle in dairy cows**



**Figure 12: Change in BFV-TAMAX (ml/min) of middle uterine arteries during an estrous cycle in dairy cows**

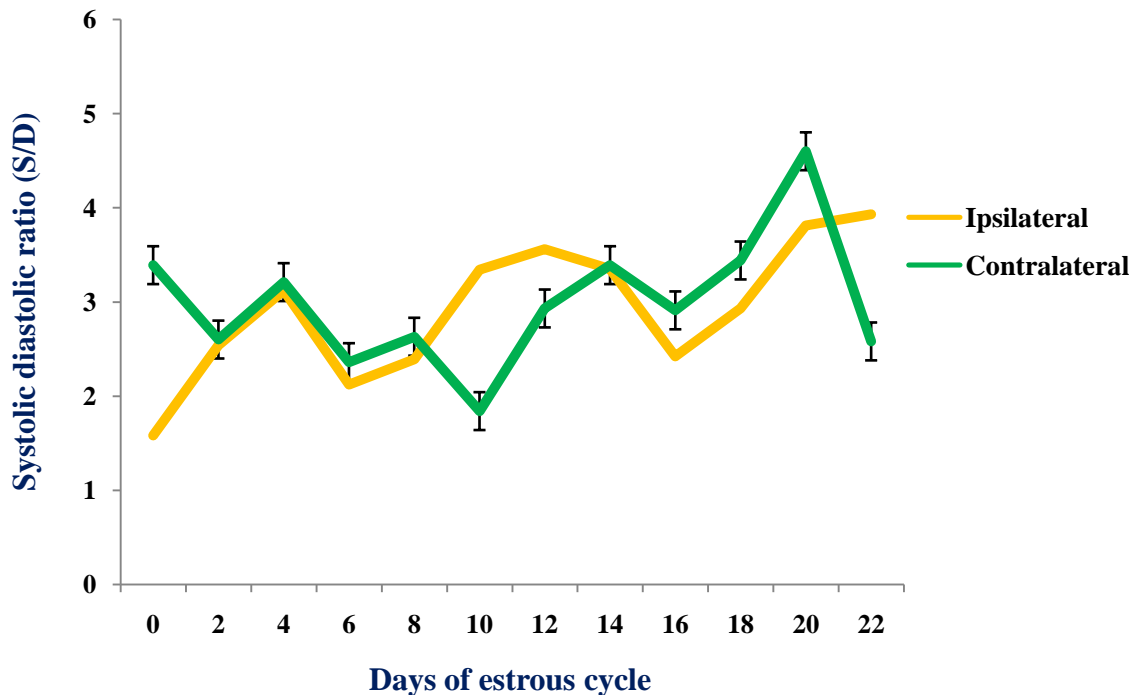


#### 4.3.7 Systolic diastolic ratio (S/D)

In present study, systolic diastolic ratio is followed a pattern similar to RI. Systolic diastolic ratio was significantly lower ( $p < 0.01$ ) in ipsilateral artery as compared to contralateral artery on day 0 i.e. day of estrus, which is mainly attributed to greater degree of diastolic blood flow (Table 5; Figure 13). However, with the progression of estrous

cycle into the luteal phase, the S/D became higher for both ipsilateral and contralateral middle uterine arteries. The S/D became non-significant ( $p>0.05$ ) except on few days when dominant follicle of anovulatory waves emerges on the ovary during the second and third wave of luteal phase.

**Figure 13: Change in systolic diastolic ratio of middle uterine arteries during an estrous cycle in dairy cows**



In conclusion, the trans-rectal ultrasonography played a pivotal role in studying the follicular and luteal development during different phases of estrous cycle. Uterine perfusion could be well understood by analyzing Doppler haemodynamic indices and useful in future study to carrying out the investigation during physiological and pathological conditions of ovaries and uterus. Augmentation of the existing diagnostic facilities with sonographic interventions is one of the many advantages it can offer.

## Chapter 5

### *Summary and Conclusions*

Present study was conducted from March, 2019 to December, 2019 at Instructional Livestock Farm complex, College of Veterinary and Animal Sciences, CSK Himachal Pradesh Krishi Vishwavidyalaya, Palampur. Ten clinically healthy cows, normal cyclic, with good genitalia and no history of reproductive abnormality were selected for present investigation. All cows were observed for the behavioural signs of estrus such as mounting, standing still while being mounted by the other cow, bellowing, increased activity, vulvar edema and cervico-vaginal discharge hanging from vulva. Following observation of estrus, cows were examined by rectal examination method. Cows with clear cervico vaginal discharge, quality uterine tone and normal genitalia were selected for the study. These cows were investigated ultrasonographically for one estrous cycle to study the development pattern of follicle(s) and corpus luteum, and, record the blood flow pattern of ovarian structures and uterus at 48 hours interval during the entire estrous cycle using brightness mode (B- mode), color Doppler mode and spectral mode ultrasonography. All the findings and various echographic structures were recorded and images were saved for further analysis and interpretation.

Out of 10 cows selected for the study, seven cows (70%) had two follicular waves, whereas, three cows (30%) had three follicular waves during one estrous cycle. Mean estrous cycle length in two-wave and three-wave estrous cycle was  $17\pm 0.31$  and  $20.67\pm 0.67$  days, respectively. For two-wave estrous cycle, first wave emerged on day  $0.43\pm 0.20$  with maximum diameter of dominant follicle reached  $12.46\pm 0.59$  mm on day  $8.57\pm 0.37$ . Emergence of second wave occurred on day  $10.28\pm 0.36$  having dominant follicle's maximum diameter of  $13.44\pm 0.56$  mm at  $17.14\pm 0.26$  days. For three-wave estrous cycle, first wave emerged on day  $0.66\pm 0.33$  with dominant follicle reaching maximum size on day  $8.00\pm 0.58$ . Following the first wave, the second and third wave emerged on day  $9.30\pm 0.33$  and  $15.33\pm 0.33$  with dominant follicle reaching maximum diameter on day  $15.67\pm 0.33$  and  $20.67\pm 0.67$ , respectively.

In two-wave estrous cycle, inter-wave duration was  $9.85\pm 0.16$  days, whereas, it was  $8.64\pm 0.33$  and  $6.03\pm 0.33$  days between first and second, second and third wave, respectively, in three-wave estrous cycle. Inter-ovulatory interval in two- and three-

follicular wave estrous cycles was  $17\pm 0.31$  and  $20.67\pm 0.67$ , respectively. Length of inter-ovulatory interval in two-wave estrous cycle was significantly shorter ( $p < 0.05$ ) than three-wave estrous cycle. Investigations revealed that in present study, two- and three-wave estrous cycle, left ovary was more active initially but with the progression of estrous cycle into the luteal phase, right ovary became more active. Thus, frequency of dominant follicles (ovulatory) in right ovary was much higher as compared to left ovary.

In present study, luteal phase has been divided into three phases i.e. early, mid and late luteal phase. In two-wave estrous cycle, mean corpus luteum diameter during early luteal phase was  $13.30\pm 0.44$  mm, showed significant increase ( $p < 0.01$ ) in diameter ( $20.85\pm 0.95$  mm), during mid-luteal phase before getting decreased non-significantly ( $p > 0.05$ ) to  $18.32\pm 0.85$  mm during late luteal phase. In three-wave estrous cycle, mean corpus luteum diameter during early luteal phase was  $11.75\pm 1.27$  mm, which rose to  $19.29\pm 0.58$  mm in mid luteal phase and remained almost same ( $19.13\pm 0.47$  mm) in late luteal phase. The significant difference ( $p < 0.01$ ) was recorded for size of CL between mid to late and early luteal phase of estrous cycle.

Vascularity of corpus luteum in two-wave estrous cycle was  $21.26\pm 1.46$ , increased significantly ( $p < 0.01$ ) to  $38.31\pm 1.46$  before decreasing significantly ( $p < 0.01$ ) to  $24.79\pm 3.15$  per cent during early, mid and late luteal phase, respectively. In three-wave estrous cycle, CL vascularity during early luteal phase was low ( $24.34\pm 4.17$  %) and increased significantly ( $p < 0.05$ ) to  $39.12\pm 1.88$  per cent in mid luteal phase and remained almost same in late luteal phase ( $39.17\pm 1.31$  %).

The significant positive linear correlation ( $r = 0.53$ ;  $p < 0.05$ ) was found between corpus luteum diameter (mm) and vascularity (%) in dairy cows. On analyzing the regression equation, a single unit increase in diameter of CL resulted in 1.42 times increase in CL vascularity during different luteal phases of estrous cycle.

Resistivity index, Pulsatility index and Systolic diastolic ratio were found to be significantly lower ( $p < 0.01$ ) in ipsilateral artery as compared to contralateral artery on day of estrus. Also, the diameter of middle uterine artery and time averaged mean and maximum velocity and was significantly higher ( $p < 0.01$ ) in ipsilateral artery as compared to contralateral artery on the day 0. Similarly, the volume of blood flow to the uterus was found to be significantly higher ( $p < 0.01$ ) on day 0 and 2 in ipsilateral as compared to contralateral uterine artery.

## Conclusions

- Two-wave estrous cycle was more prevalent in dairy cows as compared to three-wave estrous cycle, although, there were non-significant differences between diameter of dominant follicle during different waves of two- and three-wave estrous cycle.
- Corpus luteum diameter (mm) and vascularity (%) were significantly higher during mid-luteal phase as compared to early and late luteal phase and not in three-wave estrous cycle.
- Significantly higher corpus luteum vascularity (%) in cows with three-wave estrous cycle was significantly higher during late luteal phase as compared to two-wave estrous cycle and resulted in successful conception subsequently.
- A significant correlation ( $r=0.53$ ) between corpus luteum diameter (mm) and vascularity (%) irrespective of follicular waves and different luteal phases was recorded.
- A significant difference between haemodynamic indices of middle uterine artery ipsilateral and contralateral to pre-ovulatory follicle was recorded on the day of estrus. However, the difference was found to be non-significant ( $p>0.05$ ) with the progression of estrous cycle into luteal phase.

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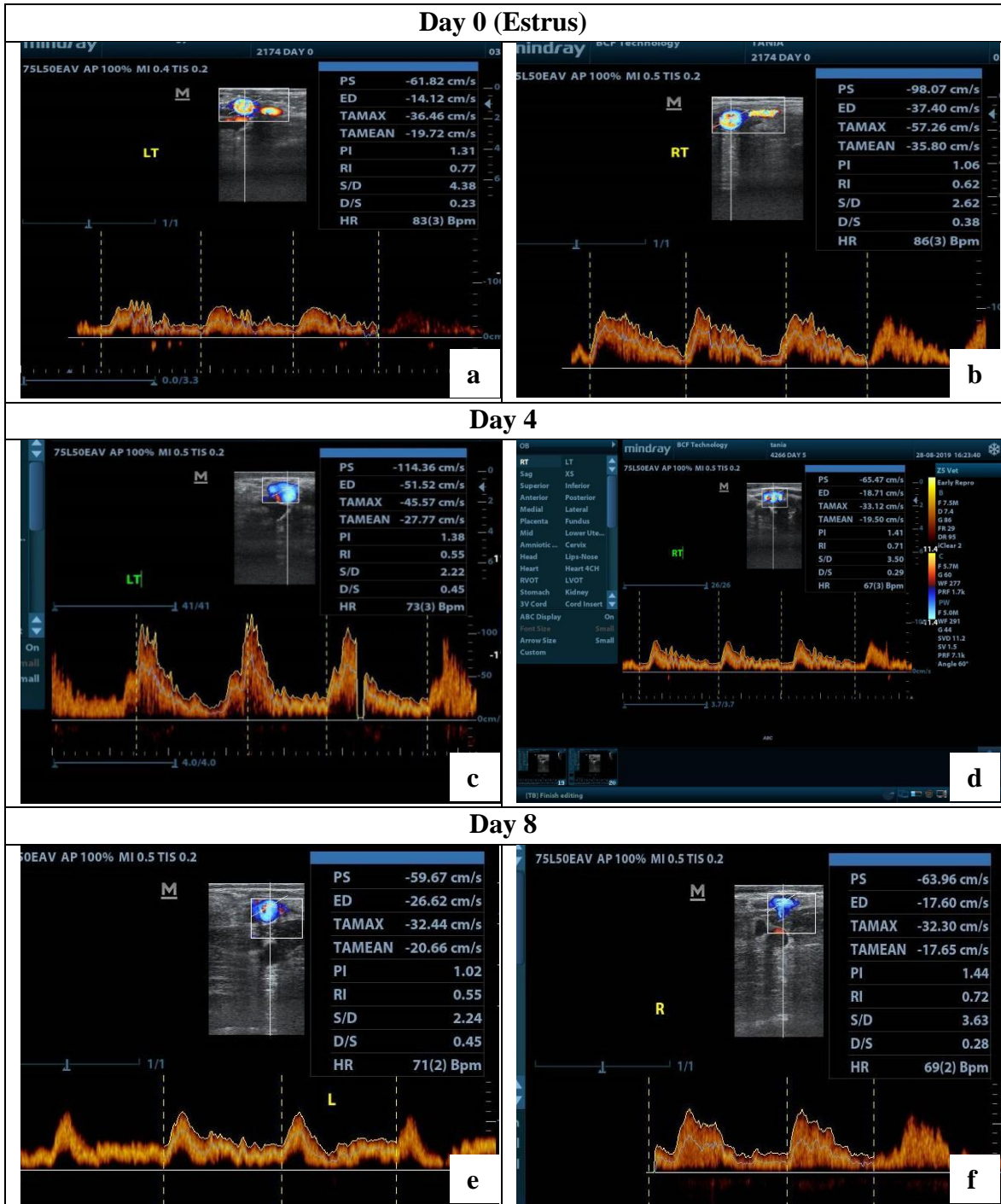
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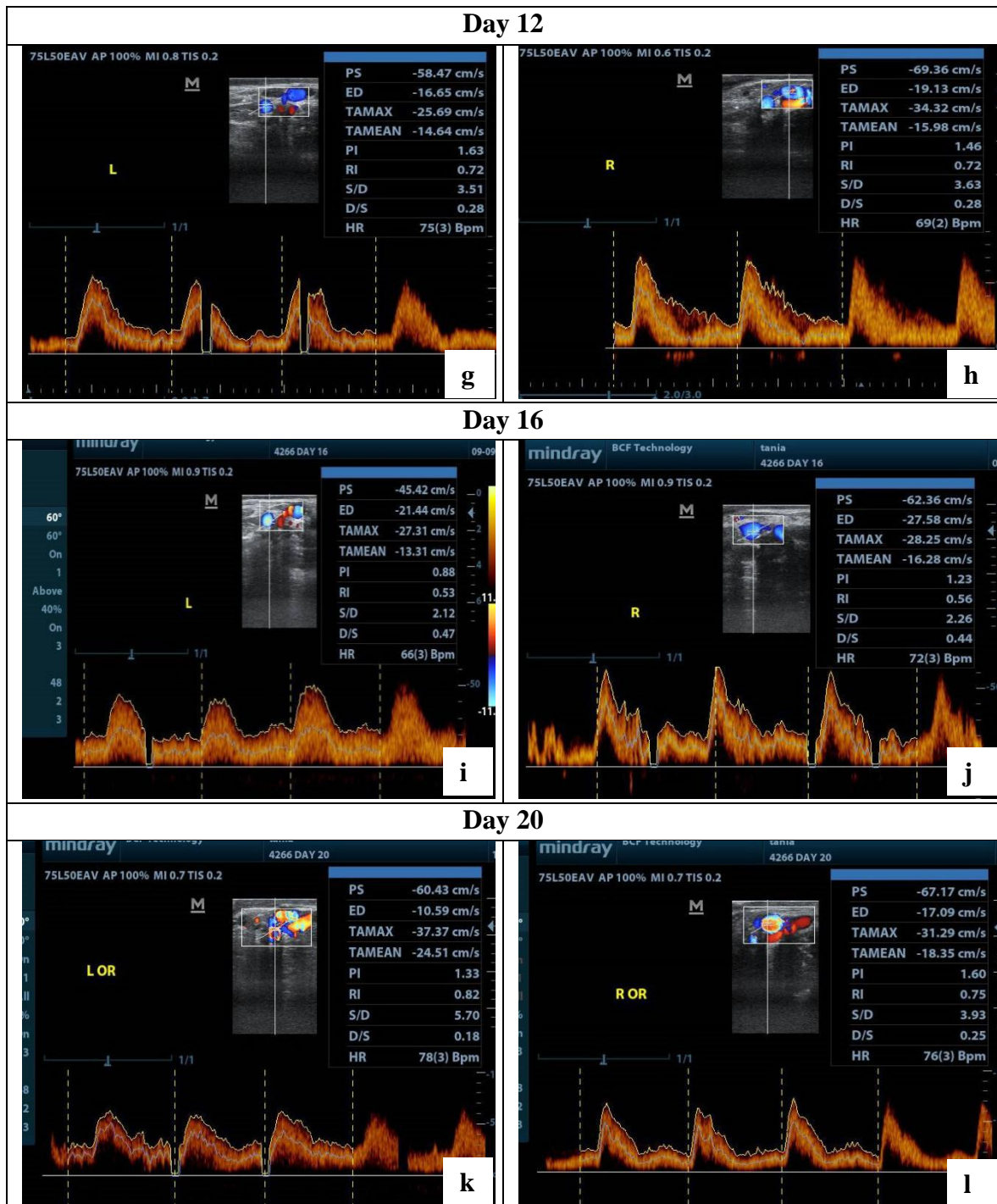
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# Appendix

## Appendix 1: Monitoring of uterine haemodynamic changes of middle uterine artery (ipsilateral and contralateral) via spectral Doppler waveform in a dairy cow during estrous cycle





Spectral Doppler waveform of middle uterine artery (MUA) in a dairy cow during an estrous cycle: **(a, b)** MUA waveform contra- and ipsi-lateral to pre-ovulatory follicle characterized by high PI, RI and low TAMEAN, TAMAX and low PI, RI and high TAMEAN, TAMAX, respectively, due to vasodilation induced by estrogen during follicular phase; **(e,f; g,h)** MUA waveform contra- and ipsi-lateral to corpus luteum spurium characterized by high PI, RI and low TAMEAN, TAMAX with the progression of cow into early and mid-luteal phase, respectively; **(k,l)** MUA waveform contra- and ipsi-lateral to corpus luteum spurium characterized by slight decrease in PI, RI and increase in TAMEAN, TAMAX with the regression of luteal tissue and beginning of new follicular phase

**Brief Resume of the Student**

**Name** : Dr. Tania Soni  
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**Academic Qualifications:**

<b>Qualification</b>	<b>Month/ Years</b>	<b>School/Board/ University</b>	<b>Marks (%)</b>	<b>Division</b>	<b>Major subject</b>
10 <sup>th</sup>	2010	HPBOSE	89.90%	First	Science, Maths, Social science, Hindi, English, IT
10+2	2012	HPBOSE	83.00%	First	Physics, Chemistry, Biology, English, IT
B.V.Sc.& A.H.	2018	Dr. GC Negi College of Veterinary & Animal sciences	70.10%	First	All courses covered under VCI curriculum

**Thesis Title in M.V.Sc:** Transrectal Doppler sonography of ovarian structures and uterus during estrous cycle in dairy cows

**Fellowships/Scholarships/Gold Medals/Awards/Any Other Distinction**

**Publications:**

**Research papers:** 1

**Scientific Popular Articles:** Nil

**Others:** Nil

**Visits abroad along with duration and purpose of visit:** Nil