OVARIAN FOLLICULAR DYNAMICS AND EFFECT OF BREED ON CONCEPTION RATE TO ARTIFICIAL INSEMINATION WITH FROZEN SEMEN IN MARES

MVSc Thesis

By

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June, 2018

Bishoftu, Ethiopia

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A Thesis submitted to the College of Veterinary Medicine and Agriculture of Addis Ababa University in partial fulfillment of the requirements for the degree of Master of Veterinary Science in Veterinary Obstetrics and Gynecology

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As members of the examining board of the final MVSc open defense, we certify that we have read and evaluated the thesis prepared by **Ararsa Duguma Benti** entitled: **Ovarian Follicular Dynamics and Effect of Breed on Conception Rate to Artificial Insemination with Frozen Semen in Mares** and recommend that it be accepted as fulfilling the thesis requirement for the degree of Masters of Veterinary Science in Veterinary Obstetrics and Gynecology

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DEDICATION

I dedicate this thesis to all group of people who work for peace and unity of Ethiopia.

SIGNED DECLARATION SHEET

First, I declare that this thesis is my original work and that all sources of material used for this thesis have been duly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for an advanced (MVSc) degree at Addis Ababa University, College of Veterinary Medicine and Agriculture and is deposited at the University/College library to be made available to borrowers under rules of the Library. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate.

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ACKNOWLEDGEMENTS

Above all, I praise the Almighty God who lead, strengthen and always keeps my life and helped me to finish this thesis successfully.

I would like to thank and express my deepest gratitude to my advisor Dr. Alemayehu Lemma for his friendly approach, constructive advice and time devotion starting from field work supervision. I also thank him for his encouragement, insight, scientific and professional guidance, critical reviews and valuable comments in writing this paper. I am very much grateful to Prof. Fekadu Regassa, Dr. Tilaye Demisse and Dr. Tamirat Degefa for their positive understanding and guidance from the beginning of my research work.

Balderas sport horses and recreational directorate of Palace administration and Holleta agricultural research center were well acknowledged for their financial and logistic support. My thanks go to Haramaya university for giving me chance to study this MSc program from the beginning. Addis Ababa university, college of veterinary medicine and agriculture was also well acknowledged and appreciated for providing me all necessary supports during my study period.

I want to express my gratitude and appreciation to Dr. Azmeraw Hibste and Mr. Girum Zewdu for their cooperation and technical assistance during field experimental work. I would like to extend my thanks to other staff members of Balderas sport horses and recreational center who involved in presenting and restraining of experimental animals while examination.

I would like to express my best regards and appreciation to my families, Chala Duguma, Fufa Duguma, Wude Nigisa, Birhanu Nigisa and Dr. Shubisa Abera for their continuous support and encouragement, specially my lovely wife Badhatu Belay for her understanding, overall moral support and caring our daughter Bilisuma in all my absence.

LIST OF ABBREVIATIONS

AI	Artificial Insemination
BCS	Body condition scores
CL	Corpus Luteum
FSH	Follicle Stimulating Hormone
GnRH	Gonadotropin Releasing Hormone
h	Hours
hCG	Human chorionic gonadotropin
IOI	Interovulatory interval
LH	Luteinizing Hormone
LOV	Left ovary
NSPC	Number of services per conception
P4	Progesterone
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
ROV	Right ovary

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ABSTRACT

Equine reproduction is unique by having long behavioral estrus and variations in ovarial follicular dynamics. Additionally, differences in time of breeding between breeds and individuals of mares made difficulty to standardize breeding time. There were a limitted data on equine reproduction and breeding in Ethiopia. An experimental study was conducted at Balderas sport horses and recreational center, Addis Ababa, Ethiopia from January to June, 2018 to determine ovarian follicular dynamics and evaluate conception rate to frozen semen in local and exotic cross breed mares. A daily transrectal ultrasonography was carried out to evaluate uterine changes and ovarian structures for 2-3 consecutive estrus cycles. Inseminations were done post ovulation within an average of 6-9h using frozen thawed semen. The mean (±SEM) of cross sectional uterine diameter were 44.4±0.5mm and 45±0.5mm for local and cross breed mares respectively; whereas endometrial fold development indicated 3.1±0.1 in local and 2.8±0.1 scores in cross breed mares with significant difference at $P<0.05$. The mean (±SEM) of preovulatory follicle diameter in local and cross breed mares indicated 49.1±1.0mm and 50.1±0.8mm respectively. Fast growth of dominant follicles in cross breed shorten interovulatory interval than local breed mares with length of estrus, 7.0±0.9 days for local and 6.1±0.6 days for cross breed mares. A positive correlation of teasing scores, uterine diameter and endometrial fold scores with diameter of developing dominant follicles in the present study has been used to estimate time of eminent ovulation. The overall conception rate to frozen semen was 15/21 (71.43%) with 8/11 (72.73%) in cross breed and 7/10 (70%) local breed mares. Conception rate increased significantly with increased number of services/conception with an overall mean ±(SEM) of 2.2±0.2 services/conception. More number of services/conception were required for local breed (2.7±0.2) than cross breed mares (1.8±0.3) and again for lower body condition scores than higher condition scores of mares. In conclusion, measuring a cross sectional uterine diameter, endometrial folding scores and teasing scores with developing dominant follicles and their correlation were good parameters to determine relative time of ovulation and breeding in mares; whereas good management of mares for improved body conditions could require to decrease number of services per conception.

Keywords: *Breeds, conception rate, follicular dynamics, frozen semen, mares, ultrasonography*

1. INTRODUCTION

Reproductive activity of equines in the northern and southern hemisphere is seasonally dependent, in which mares are polyestrous, seasonally long-day breeder (Ataman *et al.,* 2000; Gerlach and Aurich, 2000). The endocrinology of the estrus cycle involves a balance between hormones produced by the pineal gland, hypothalamus, pituitary gland, ovaries, and endometrium (Malpaux *et al.,* 2001; Aurich, 2011; Busato *et al.,* 2017). The hypothalamus produces gonadotropin releasing hormone (GnRH), which is released in brief pulses into the hypothalamic-pituitary portal system, and stimulates the synthesis and release of the gonadotropins, the follicle stimulating hormone (FSH) and luteinizing hormone (LH), from the anterior pituitary gland for follicular growth, maturation and ovulation (Malpaux *et al.,* 2001; Raz and Aharonson-Raz, 2012). In tropical areas seasonal fluctuation causes an increase or decrease in body condition of animals as a result of variation in feed resources, and ovarian activity was also closely following this seasonal pattern (Lemma *et al.,* 2006a). This confirms reproductive behavior in tropical area are affected by forage availability than length of light per day (Lemma *et al.,* 2006a; Scharf and Carroll, 2010; Delgadillo, 2011).

The pattern of ovarian follicular dynamics during estrous cycle in mare vary between breeds under different environmental condition (Ginther, 2000; Shirazi *et al.,* 2002; Davies Morel *et al.,* 2010; Camargo *et al.,* 2017; Najjar *et al.,* 2018). The maximum diameter of the preovulatory follicle at 42.6 $\pm$1.24 mm in Caspian mares (Shirazi *et al.,* 2002), 39.95 $\pm$ 4.84 mm in Thoroughbred mares of the United Kingdom (Davies Morel *et al.,* 2010), 38.8 $\pm$ 0.6 mm in mixed breeds of Pony mares (Gastal *et al.,* 2004), 29.4±9.19 mm in Balderas sport horses of Ethiopia (Lemma *et al.,* 2015), showed variable reports and is still difficult to identify the precise time of ovulation and breeding, as every mare do not ovulates with strictest expectations of time. In general, when the pre-ovulatory follicle reaches 35 mm, ovulation can occur within approximately 36-48h; though this varies considerably between individual mares (Patricia, 2018).

The use of biotechnologies in equine reproduction has significantly increased in recent years. Among these, artificial insemination (AI) has been shown to result in reliable results as this technique also favors the use of superior genetic material to improve the genetics of equine herds

(Vianna, 2000; McCue, 2012). The time of insemination relative to ovulation is critical for both proper fertilization, and higher pregnancy rate, due to the short span of the viability of ovum and sperm in female reproductive tract. The longevity of ejaculated sperm within the female reproductive tract is 48 h, whereas the longevity of egg after ovulation is <12 h; which support the reason why horse breeders usually inseminate mares prior to ovulation (Ginther, 1992; Yoon, 2012). Woods *et al.* (1990) evaluated an equivalent rate of pregnancy to frozen semen when mares are inseminated up to 12 h post ovulation and pre-ovulatory insemination. Similarly, Sieme *et al.* (2003) also recommended single insemination with frozen semen should preferably be carried out between 12 h before or 12 h after ovulation.

The timing of ovulation in mares is usually anticipated by measuring the size of a dominant follicle, the structural changes of the uterus and cervix, as well as estrus related behavior of the animals (Stout, 2003; Samper, 2008). The overt signs of estrus are attributable to the estrogen production by the follicle on the ovary. As a mare exhibits estrus, estrogen causes a swelling and increased folding in the endometrium (Pycock, 2002). Uterine diameter and endometrial folds become more prominent with increasing diameter of developing dominant follicles as ovulation time closer (Lemma *et al.,* 2006b). This has been used to estimate time of eminent ovulation.

A number of factors play a role in poor reproductive performance of mares which includes, aging, stress, inbreeding, reproductive disorders, fertility of male or female, nutritional problems, and hormonal imbalance. Among these factors aging have an influence on preovulatory follicle diameter, which is known to be associated with decreasing reproductive competence (Davies Morel *et al*., 2010); changes in the uterus and hormones (Ginther, 1993). Additionally, dose of semen based on semen type used have an effect on success of pregnancy rate to AI (Vidament *et al.*, 1997; Pickett *et al.*, 2000; Mustafa *et al.*, 2016). A significant effect of pre or post ovulation artificial insemination on pregnancy rate have been demonstrated by different researchers (Woods *et al.*, 1990; Samper, 1997; Brinsko *et al.*, 2010). The side of ovary with ovulatory follicle (Najjar, *et al.,* 2018), and increased number of cycles per pregnancy, when mares are bred with frozen-thawed semen compared to natural or fresh semen are also influential factors on fertility rates (Squires *et al.,* 1999).

There were a limitted data on equine reproduction and breeding in Ethiopia; however previous studies by Lemma *et al.* (2015) indicated high early embryonic loss that might be due to the effect of inbreeding at Balderas sport horses of Ethiopia. The long periods of behavioural estrus (5-7 days on average), variations in uterine and ovarial follicular dynamics and differences in time of breeding between breeds and individual mares, could confirms the need for study of ovarain follicular dynamics in specific breeds to determine relative time of ovulation and breeding for reproductive efficiencies. Therefore, in this document it is hypothesized that breed determines the difference in ovarian follicular dynamics and conception rates to AI from frozen semen.

Objectives:

- To determine ovarian follicular dynamics in local and exotic cross breed mares
- To evaluate conception rate to artificial insemination with frozen semen in local and exotic cross breed mares
- Determine effect of breed and other animal related factors on conception rate to AI with frozen semen

2. REPRODUCTIVE PHYSIOLOGY OF MARES

Reproduction in equine species is strongly influenced by the photoperiod, where the reproductive season occurs in the months of long days (spring and summer in northern hemisphere) (Gerlach and Aurich, 2000; Nagy *et al.,* 2000). Between the reproductive season, or cyclic phase, and the anestrus or acyclic phase, there are two transitional periods (spring and autumn transition), which are marked by important peculiarities in the physiology of the mares (Ginther, 1992). In fillies, puberty occurs approximately at an age of 12-18 months (Aurich, 2011); but this age is again influenced by season. In fillies born very early in the given year, puberty will not occur before the beginning of the breeding season of the following year. Senescence in old mares is rarely seen and most mares continue to cycle independent of age. However, in old mares, the interovulatory interval (IOI) may be longer than in young and middle-aged mares due to a slower growth rate of the dominant follicle (Ginther *et al.,* 2008b).

The endocrinological control of the estrus cycle is governed by the hypothalamic-pituitary-gonad axis and represents the basis for the study of reproductive physiology that produce ovulatory estrus cycles in the mares (Malpaux *et al.,* 2001; McKinnon, 2011; Busato *et al.,* 2017). The major hormones involved in this axis include GnRH, FSH, LH, estrogen, inhibin, and progesterone (Roser, 2008). Overriding the whole of this control mechanism is the effect of photoperiod; decreasing day length causing estrus cycles to cease and increasing day length causing them to occur (Davies Morel, 2003).

2.1. Estrus cycle in mare

Mares are seasonally polyestrous and cycle when the length of daylight is long (Ginther, 1992; Nagy *et al.,* 2000). During short length of daylight mares are in state of anestrus, in which the uterus is flaccid, and the ovaries are inactive with no significant follicles or corpora lutea. The cervix may be closed but not firm and tight, or it may be thin, short, and dilated. As the length of daylight increases, mares undergo a vernal transition and the ovaries become active, with numerous large (>25mm) follicles (Patricia, 2018). The end of vernal transition is marked by a surge of luteinizing hormone and subsequent ovulation. After this ovulation, the first 21 day interovulatory period of that breeding season occurs and a regular estrus cycle is established (Aurich, 2011; Patricia, 2018).

The equine estrus cycle is commonly described as a combination of a follicular phase, or estrus, and a luteal phase, or diestrus (Crowell-Davis, 2007). Estrus, or follicular phase is characterized by the presence of follicles at different stages of development, and has duration of about 5-7 days. During this period, mares are receptive to a stallion, cervical relaxation, presence of a large (dominant) follicle, and endometrial edema (Samper, 2008). Mares in estrus raise their tail, squatting with legs spread apart, urinate, evert the vulvar lips to expose the clitoris, and ultimately tolerate copulation (if a stallion nears, she may lean into him) (Samper, 2008; Patricia, 2018).

Diestrus is characterized by lack of stallion receptivity, presence of a corpus luteum (CL), a tight cervix, and lack of endometrial edema (Samper, 2008). This phase of estrus cycle is dominated by progesterone (P4) (concentrations >1 ng/mL) produced by the corpus luteum. The diestrus period ends with regression of the CL which occurs due to prostaglandin F2α ($PGF_{2\alpha}$) released from the endometrium of the non-pregnant mare (Crowell-Davis, 2007; Ginther *et al.*, 2008a). Unlike the follicular phase the insensitivity of the CL to photoperiod makes the length of this period more constant and estimated as an average duration of 14-15 days, but can be more durable in mid-summer (16 days) than in spring or autumn (13 days) (Ginther *et al.*, 2008a; Raz and Aharonson-Raz, 2012).

The mares may present one or two follicular waves per estrus cycle, being more common is the occurrence of a single wave (Ginther, 1992; Ginther, 2000). In the ovulatory waves the dominant follicle enlarges and then softens just before ovulation in which the oocyte is released through the ovulation fossa. A corpus hemorrhagicum and subsequent corpus luteum form and produce P4, which stimulates closure of the cervix and an increase in uterine tone (Yoon, 2012; Patricia, 2018).

During follicular growth, approximately 7-11 follicles per wave emerge at diameters of 5-6 mm and enter a common-growth phase of about 6 days, in which follicles grow at an approximately similar rate and each follicle has the capacity for future dominance (Gastal *et al.*, 1997; Aurich, 2004). Subsequently, the deviation process occurs, in which several intrafollicular factors increase in the largest follicle of the ovulatory wave but not in the subordinate follicles; and only the dominant follicle continues to grow (Beg and Ginther, 2006). The deviation in growth of follicle between the two largest follicles occurs soon after FSH levels decrease. The intrafollicular changes in the future dominant follicle apparently increase the responsiveness of this follicle to decreasing FSH and increasing LH concentrations which occur at that time. The largest follicle alone continues to grow, becomes dominant, and eventually ovulates (Gastal *et al.,* 1997; Aurich, 2004; Raz and Aharonson-Raz, 2012).

Ovulation in mare is process of complex series of events under elevating LH concentrations that lead to rupture of a preovulatory follicle at the ovulation fossa and the extrusion of follicular fluid, granulosa cells, and the cumulus oocyte complex which usually occurs 1 or 2 days before the end of estrus (Gastal and Gastal, 2011). The unique anatomical structure (ovulation fossa) of the ovaries of mares is known to limit the number of eggs ovulated (Yoon, 2012). The cells, of the granulosa remaining at the ovulation site, undergo a process of luteinization by the action of LH in developing primary CL and modifies the secretory activity of granulosa cells, which cease to secrete estradiol and replaced by P4 secretion until initiation of luteolysis at around day 14 post-ovulation. The mare is characterized by less concentration of ovulatory LH surge but a progressive increase in LH lasting many days, with a maximum concentration after ovulation (Bergfelt and Adams, 2007; Norman and Larsen, 2010). If pregnancy does not occur, lysis (destruction) of the CL occurs due to endogenous release of prostaglandin from the uterus starting at about 13 or14 days post-ovulation, thus causing a fall in the P4 level, and the cycle starts again (Crowell-Davis, 2007; Ginther *et al.,* 2008a). At about the same time (day 13), FSH levels will increase causing the follicle to grow in preparation for ovulation of the following estrus (day 19 to 23) (Slusher *et al.,* 2004).

2.1.1. Influence of photoperiod on equine reproduction

Species of animals with presentation of puberty and reproductive activity under long photoperiod, experience an increase in the number of hours a day with low concentrations of circulating melatonin. Melatonin is synthesized and secreted mainly by the pineal gland, under a neuronal control system in which the perception of light in the retina blocks the synthesis and secretion of pineal melatonin (Malpaux *et al.*, 1996). The photoperiod exerts influence on the secretory pattern of melatonin, a hormone responsible for regulating the hypothalamic production of GnRH

(Malpaux *et al.,* 2001). In equines, during the months of negative photoperiod (short days) the increase in melatonin levels leads to a reduction in GnRH secretion (Nagy *et al.*, 2000; Aurich, 2011).

The main stimulus for the initiation of cyclicity in the mare is the increase in the hours of daily luminosity. In this species, the decrease of melatonin secretion by the pineal gland (positive photoperiod) initiate cyclicity. The Mechanism behind the effects of photoperiod involves the secretion of melatonin, which stimulates GnRH release in short day breeders and inhibit GnRH production in long day breeder animals (Malpaux *et al.*, 1996; Aurich, 2011). The retina of the eye is stimulated by the presence of light and when light is abundant, an excitatory pathway is active inhibiting melatonin release, but increase in melatonin secretion occur when day length shortens or extended darkness. Because of genetic differences, different breeds of animals maintain different levels of photo responsiveness (Ginther, 1992). This is one reason why differences of breeding time occur between different breeds. GnRH is secreted from the hypothalamus in response to long day light and decreased melatonin concentrations in mares (Smith *et al.*, 2008).

Since mares are classified as long-day seasonal polyestrus, they are known to have two well-defined reproductive seasons (namely: cyclic or fertile season and acyclic or anestrus season) throughout the year due to the influence of photoperiod (Busato *et al.*, 2017). In cyclic season, follicular development is followed by ovulation and formation of functional CL, occurs from spring to summer. The anestrus season, or ovarian quiescence, predominates during autumn to winter. Between the cyclic and anestrus phases, there are two transition periods termed as spring transition and autumn transition. The spring transition occurs between the end of the anestrus season and the beginning cyclic season since the autumn transition occurs at the end of the breeding season and beginning of the anovulatory phase (Ginther, 1992; Busato *et al.*, 2017). During the anovulatory season, there are mares that present follicular development accompanied by characteristic estrus; however, ovulation does not occur, leading to low pregnancy rates during winter and early spring.

Reproductive seasonality consists of the phenomenon by which some species have reduced sexual activity during a certain period of the year, mainly regulated by climatic factors such as temperature, air humidity, or photoperiod (Coloma *et al.,* 2011). In tropical climates, high ambient temperature observed in the dry season is one of the main limiting factors for reproductive efficiency (Chemineau *et al.,* 2006; Scharf and Carroll, 2010). In females, exposure to stressors such as high temperatures, feed, and water deprivation can act on the hypothalamic-pituitary-gonadal axis, jeopardizing their operation (Delgadillo, 2011). Feed and water deprivation reduce the release of GnRH from the hypothalamus and determine a decrease in the frequency and amplitude of LH, resulting in abnormal ovarian function and thus inhibiting follicular development and ovulation (Dobson *et al.,* 2001; Delgadillo, 2011). The nutritional deficiency is the main factors that can interfere with reproduction of females in tropical regions, since the energetic support is usually not enough to maintain the production processes in the dry period (Silva and Araujo, 2000). A lower ovulatory rate during the dry season when there are more light hours per day were reported in equines of tropical regions. BCS were strongly correlated to the number and size of ovarian follicles. The improvement in body condition has generally, a positive impact on emergence of small growing follicles there by having influence on the total count of ovarian follicles than length of light per day in tropical regions (Lemma *et al.,* 2006a).

2.1.2. Hormonal regulation of estrus cycle in mare

The endocrinology of the estrus cycle involves a delicate balance among hormones produced by the pineal gland, hypothalamus, pituitary gland, ovaries, and endometrium in equine reproduction (Malpaux *et al.,* 2001). Melatonin is released during the hours of darkness, following a pattern of secretion inversely proportional to the amount of daylight hours. As a result of increased exposure to photoperiod in the spring and summer, the secretion of melatonin decreases, which in turn stimulates the release of GnRH by hypothalamus in the mare (Malpaux *et al.,* 1996; Smith *et al.,* 2008).

During estrus cycle, GnRH stimulates the production of the two important gonadotropins FSH and LH (Donadeu and Ginther, 2001; Malpaux *et al.,* 2001). Both gonadotropins are transported through the blood to the ovary where specifically exert their functions. FSH acts on the granulosa cells of the preovulatory follicle and stimulating the growth, follicular maturation and estrogen biosynthesis. On the theca cells, LH is involved in oocyte maturation, ovulation, establishment and maintenance of CL as well as development and in the synthesis of P4. Both ovarian steroids control the hypothalamus-hypophysis axis by feedback mechanisms that determine the estrus cycle in the mare (Evans *et al.*, 2011; Velez *et al.*, 2012). FSH plays a key role in the emergence of the ovulatory wave, but the plasma circulating FSH declines when the size of the largest follicle reaches time of divergence (Gastal *et al.*, 1997; Donadeu and Ginther, 2001). It seems likely that the largest follicle has ability to utilize the low level of FSH, while other factors such as LH, inhibin, insulin-like growth factor 1 (IGF-1) and estradiol also play a role during follicle deviation (Bergfelt *et al.*, 2001).

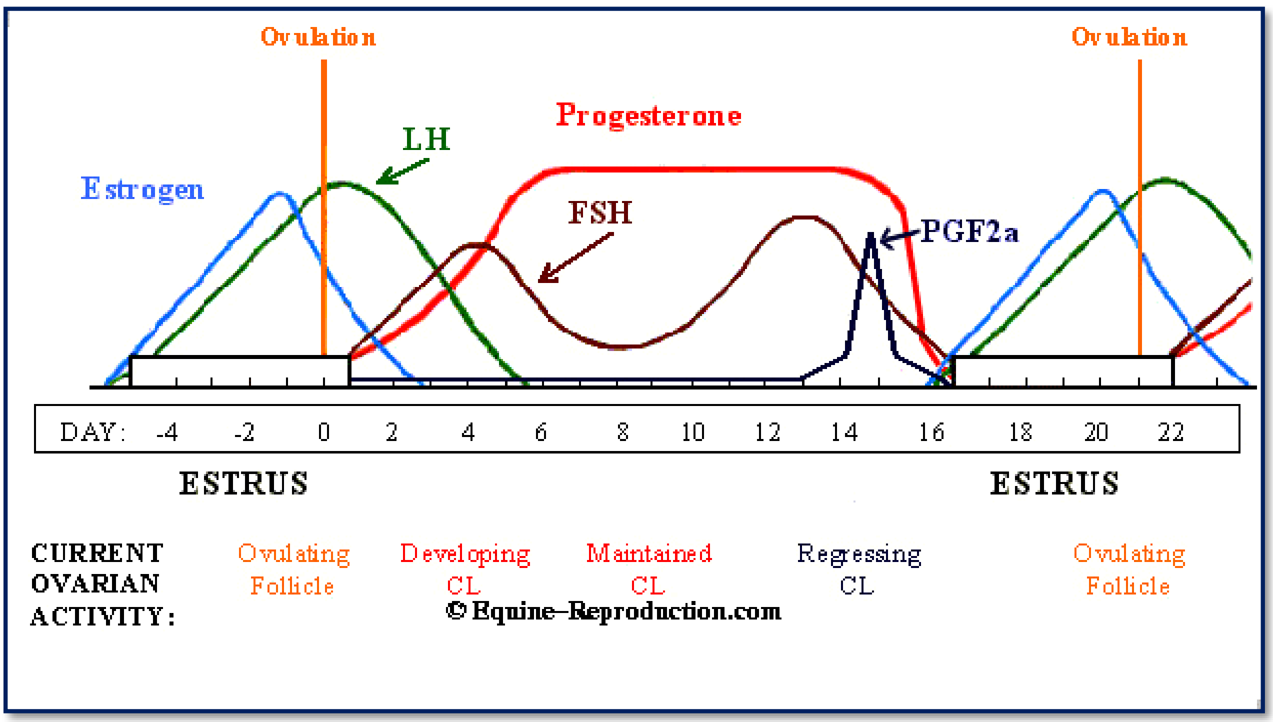


Figure 1: Hormone levels and corresponding ovarian activity in the estrus period

Source: Slusher *et al.* (2004)

Blood levels of estrogen are highest when the mare is in estrus. Estrogen is responsible for causing the mare to show the behavioral signs of receptivity to a stallion. FSH is mainly responsible for ovarian follicular development (Gastal *et al.*, 1997; Samper, 2008). P4, which is produced by the corpus luteum, is the primary hormone that is responsible for the recognition and maintenance of early pregnancy up to about 150 days in mare. From approximately 150 days until term, the source of progestins for pregnancy maintenance is the placenta (Slusher *et al.,* 2004).

Luteinizing hormone is responsible for stimulating ovulation and supporting the initial stages of corpus luteum development. The pattern of LH secretion in the mare differs from that of other domestic animals. LH is secreted for a prolonged period of time, beginning at the initiation of behavioral estrus, peaks at 1-2 days after ovulation and declines during the early luteal phase (Jones and Troxel, 2006; Norman and Larsen, 2010). The LH level at the time of LH peak is lower than most other species. P4 realizes negative feedback on the release of LH, causing the levels of this hormone to remain low during the diestrus. In mares, secretion of P4 begins on the day of ovulation, and blood concentration reaches maximum values after 6 days (Ginther *et al.,* 2006; Busato *et al.,* 2017).

Prostaglandin is fatty acid hormone produced naturally by many tissues of the body, but the types produced by uterine lining and endometrium is concerned in reproduction. It induces luteolysis which consists of functional and structural regression of the CL by decrease in secretion of P4 and interrupt the luteal phase (McCue, 2007; Squires, 2008). PGF is not effective in animals that do not have a mature CL including prepubertal heifers, postpartum anestrus mare, or cycling females in the first five days of the estrus cycle. The PGF reaches the ovary via systemic circulation and triggers the lysis of CL around 14 days of the estrus cycle in mare (Ginther *et al.,* 2006).

2.1.3. Follicular dynamics during estrus cycle in mare

The cortical structures of ovary consist the follicles in which each ovum is encased by a single layer of follicular epithelial cells, the primordial follicle (Jones and Troxel, 2006). There are approximately 35, 000 primordial follicles in the mare ovary at birth, of which 100 are maturing at any given time (Youngquist and Threlfall, 2007). Only one and sometimes two follicles ovulate during each estrus cycle and therefore the great majority of follicles undergo atresia (Norman and Larsen, 2010).

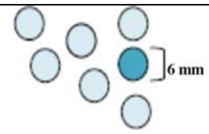
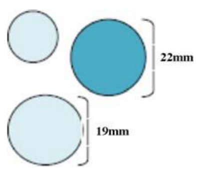
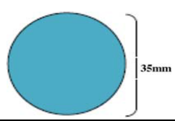
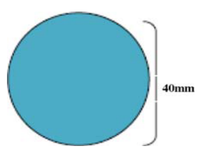
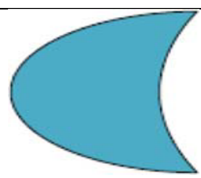
Follicular dynamics is the process of continual growth and regression of antral follicles that leads to the development of the preovulatory follicle. The exact dynamics of the estrus cycle differ between mares and cycles, however aspects of the cycle (e.g. maximum follicle diameter) are known to be quite repeatable within individual mares (Ginther *et al.,* 2008c). The mean size of the preovulatory follicle just prior to ovulation has been reported to be 40 mm, with a very large range of 30 to 70 mm (Bergfelt and Adams, 2007), and follicular growth rate is reported as approximately 3 mm a day until one-day pre-ovulation (Aurich, 2011).

The follicular growth process involves enlargement, development of cellular layers around the follicular wall, accumulation of fluid within the central follicular cavity and production of the hormone estrogen (Bergfelt *et al.,* 2001; Conti *et al.,* 2006). As the enlarged preovulatory follicle nears maturity, it bulges from the ovary's surface. This bulge can be felt through the rectal wall when the mare's ovaries are manually palpated. Ultrasonic images of the preovulatory follicle appear as a black area within the grayish tones of surrounding ovarian tissues (Brinsko *et al.,* 2010). The follicle's diameter indicates its maturity with 35 mm or greater that is considered as capable of ovulating. Another cortical structure, the corpus luteum, forms from the tissues remaining after a follicle ruptures at ovulation. Unlike the follicle, the corpus luteum is solid-cored and secretes the hormone P4 (Jones and Troxel, 2006).

Ovarian follicle development in the mare is characterized by waves of several follicles that emerge and initially grow in synchrony. Various numbers and types of follicular waves develop during an equine IOI (Ginther, 1995). Follicular waves in mare can be classified as major and minor. Major waves are those in which follicular divergence occurs, with the formation of dominant and subordinate follicles. In the minor waves there is no divergence and, consequently, there is no formation of dominant follicles or ovulation. Major waves can be primary or secondary waves. The primaries emerge during the diestrus and result in primary or estrus ovulations. The secondary ones emerge during early estrus or diestrus and give rise to anovulatory follicles or result in ovulation during diestrus (Ginther, 1993; Patricia, 2018). The follicular waves emerge at 8 to 10 day intervals resulting in two follicular waves during each estrus cycle involving different cohorts of follicles (Ginther, 1993). Following follicular recruitment, the primary wave of follicular growth is characterized by an initial increase in the number of follicles greater than 10 mm and this period is described as the common-growth phase and continues for a mean of 7.4 ± 0.5 days or until approximately 6 to 7 days before the next ovulation (Ginther, 1992; Ginther, 1993). Usually, one follicle becomes dominant and ovulates when it is ≥30 mm in diameter (Patricia, 2018). During its growing phase dominant follicle suppresses not only its subordinates but also the emergence of other waves and causes regression of the static dominant follicle of the preceding wave (Ginther, 1993).

During follicular development, the day of emergence of a follicle is defined as the day before the follicle first exceeds 6 mm (Gastal *et al.,* 1997), while the day of wave divergence will be defined as the point after which differentiation of follicle in growth rates between the dominant and largest subordinate follicle. The duration of growth of a follicle is the time taken by that follicle to grow from 6 mm in diameter to its maximum diameter. The growth rate can be calculated by dividing the difference between maximum and minimum diameter of the dominant follicle by the duration of its growth (Gastal *et al.,* 1997). Interovulatory intervals are classified into those that had 1 wave (ovulatory wave) and 2 waves (anovulatory wave during the first half of the IOI and an ovulatory wave during the second half). The dominant follicle of one-wave intervals and the dominant follicle of the second wave in two-wave intervals is described as the dominant ovulatory follicle and the other follicles that appeared to originate in a given wave is defined as subordinates (Ginther, 1993).

Table 1: Follicular development during the estrus cycle of the mare

Day of cycle	Size of the largest follicle	Stage of the cycle	Hormone concentration in peripheral circulation	Occurrence within the (later) dominant follicle
7		Development of the follicular wave	• FSH↑	•Emergence • Growth (all follicles of the wave)
13		Follicle deviation	• FSH↓ • Oestradiol-17β↑ • Inhibin↑	•Continued growth (3mm per day) Activation of deviation mechanisms: •Sensitivity to FSH ↑, Free IGF-I ↑, Inhibin ↑, Oestradiol ↑
17		Beginning of the preovulatory phase	• LH↑ • Oestradiol-17β ↑	•Continued growth (3mm per day)
19		Preovulatory phase	• LH→ • Oestradiol-17β↑	•Stagnation of growth •Maturation (characteristic pattern in the expression of different factors, e.g. PGE ↑, $PGF_{2\alpha}$ ↑, prostaglandin dehydrogenase ↑)
21		Ovulation	• LH↑ • Progesterone↑	•Granulosa cells→luteinization •Progesterone increase • Vascularization

Source: Aurich (2011)

Emergence of the dominant follicle is referred to as deviation and is preceded by an increase in IGF-1, oestradiol, inhibin-A and activin-A in the future dominant follicle (Beg and Ginther, 2006). Inhibin produced by the granulosa cells of the dominant follicle inhibits continued growth of other follicles in the same cohort as it causes decline in production of FSH. The process of follicular deviation begins when the two largest follicles in any cohort have a mean of 22.5 and 19.0 mm in diameter respectively (Ginther, 1993; Gastal *et al.*, 2004).

Ovulation is a physiological process with an inflammatory component that depends on the coordinated activity of gonadotrophins and steroid hormones, as well as inflammatory mediators such as cytokines, prostaglandins, leptin, nitric oxide and matrix metalloproteases (Conti *et al.*, 2006). The LH surge causes major re-modelling of the ovarian follicle in preparation for the ovulatory process (Conti *et al.*, 2006).

Breed difference of ovarian follicular dynamics

Differences in some aspects of ovarian follicular development between different breeds of mares need determination of growth pattern for the specific breeds to improve reproductive efficiency. The Arabian mares began estrus with a smaller mean follicle diameter of 29.5 ± 1.1mm than Quarter horse mares 33.3 ± 1.2mm (Najjar *et al.,* 2018). The emergence of the preovulatory follicles occur earlier in mares with 1 major wave than in mares with 2 major waves per cycle (day 6.3 ± 0.9 versus day 11.0 ± 1.2) (Shirazi *et al.*, 2002). In some breeds (quarter and ponies), usually only one major wave develops in the late diestrus and culminates at the time of estrus, whereas, in other breeds (thoroughbreds), a secondary major wave may develop in late estrus or early diestrus, and the dominant follicle may be ovulatory or anovulatory during diestrus (Ginther, 1993; Ginther, 2000). The primary waves originated on day 6.4 ± 0.81 (ovulation-day 0) when the mean diameter of ovarian follicles was 9.6 ± 1.05 mm. Divergence between the dominant preovulatory follicle and subordinate follicles occurred on day 13.4 ± 0.81, when the dominant follicle was 18.1 ± 2.67 mm in diameter. The largest follicle in horse is 22.5 mm at the beginning of deviation (Ginther *et al.,* 2004). The intervals from emergence to divergence and from divergence to ovulation were 7 ± 0.68 and 8.7 ± 0.68 days, respectively; with the IOI of 22.1 ± 0.43 days (Shirazi *et al.*, 2002).

After ablation of all follicles of ≥6mm 10 days after ovulation, the interval of emergence at 6.0-6.9 mm for different follicles were 1.3 to 3.2 days. The first six days after follicle ablation are days of common growth phase, whereas the day of expected deviation is 12.4 ± 0.6 when follicles reach ≥20mm. During the interval from expected deviation to ovulation, there were length of the interval 7.7 ± 0.2 days in mixed breeds of pony mares (Gastal *et al.,* 2004). Follicular deviation is preceded by a common growth phase of several days. During this phase, the follicles grow at an approximately similar rate and each follicle has the capacity for future dominance (Gastal *et al.*, 2004; Beg and Ginther, 2006).

The average diameter of the preovulatory follicle was 45mm in lactating mares of Criollo breed in southern Brazil (Camargo *et al.*, 2017). Mares with preovulatory follicle diameter of approximately 35-44 mm in Arab pure breed mares provides good pregnancy rate (Najjar *et al.*, 2018). Dolezel *et al.* (2012), reported the preovulatory diameter of 48 mm in Czech warmblood mares, Ginther and Pierson (1989) with preovulatory follicle size of 46 mm and 48 mm at different season of study. Other researchers like Dimmick *et al.* (1993), Shirazi *et al.* (2002), Ginther *et al.* (2004), Ginther *et al.* (2008c) reported preovulatory follicle diameter of 40-45mm on the day before ovulation. The difference in diameter of preovulatory follicles might be affected by breed, season of the study (Ginther and Pierson,1989; Dolezel *et al.*, 2012), number of ovulation per cycle (Ginther *et al.*, 2008c), as well as age of animals as confirmed by Davies Morel *et al.* (2010). The preovulatory follicle size in single ovulators and double ovulators in mare made significant difference with diameter of 40.6 $\pm$1.0 mm and 36.5 $\pm$ 0.6 mm respectively (Ginther *et al.*, 2008c). The concentration of FSH were significantly lower in the double ovulators which was attributed to greater output of estradiol by the two dominant follicles; but Plasma LH concentrations did not differ between single and double ovulators until after ovulation (Ginther *et al.*, 2008c). It is possible that the smaller follicle size at ovulation may be related to body size of mares (Dimmick *et al.*, 1993).

2.1.4. Manipulation of estrus cycle in mare

Efficient horse breeding programmes require synchronization or scheduling of estrus, and ideally ovulation, in the mare. Manipulation of ovarian function allows the timing of insemination to closely coincide with ovulation thereby improving pregnancy rates (Norman and Larsen, 2010). The main advantages of estrus or ovulation synchronization and AI in mares are to have one service per cycle for high-demand stallions, scheduled breeding for mares transported to stallions, reduced uterine contamination, synchronization between donor and recipient mares in embryo transfer programs; reduced labor and veterinary costs (Samper, 2008). Hormonal imbalance occurs during transitional period with high FSH and low LH secretions. Thus, hormonal treatment during the transitional period is to hasten initial ovulation of the breeding season and to suppress the long erratic estrus period (Ataman *et al.*, 2000; Lemma *et al.*, 2015).

Estrus synchronization in horses has been problematic because of the long duration of behavioral estrus and the variable time frame to ovulation. Options for estrus synchronization include the use of P4, one or two injections of $PGF_{2\alpha}$, and follicular ablation for manipulation of the reproductive cycle in mares (Bergfelt and Adams, 2007; Klug and Jochle, 2001).

Prostaglandin

In the non-pregnant, cycling mare, prostaglandins are produced and secreted in pulses by the endometrium from approximately day 13 to 15 post-ovulation (Bergfelt and Adams, 2007). Prostaglandin used for induction of estrus, estrus synchronization, treatment of mares with a maintained CL, induction of uterine contraction for evacuation of fluid, and induction of abortion (Squires, 2008). In the mare, $PGF_{2\alpha}$ reaches the ovary via the main circulatory system, not by a local counter-current transport system as seen in the ewe and cow. The products most commonly used in horses are Lutalyse® and Estrumate®. Estrumate® has less of a tendency to cause side effects such as mild sweating and abdominal discomfort (McCue, 2007).

Administration of $PGF_{2\alpha}$ to a mare in diestrus causes luteolysis and allows a follicle to grow, mature and ovulate. The corpus luteum must be 5-14 days old to respond to $PGF_{2\alpha}$ and mare will come into estrus 2-5 days after administration. Time to ovulation is variable (3-10 days) and depends on the stage of the mare's current follicular wave and on the size and character of follicles at the time of $PGF_{2\alpha}$ administration (Patricia, 2018). Prostaglandin is effective only during the luteal phase of the estrus cycle and is less effective in causing luteolysis if given prior to five days after ovulation. The specific mechanism for this reduced efficacy on the immature CL is due to reduced receptors and luteolytic enzymes within the CL (Norman and Larsen, 2010).

Ovulation induction

In many species, excluding the mare, ovulation is preceded by a surge in LH in the systemic circulation known as the 'pre-ovulatory LH peak' (Bergfelt and Adams, 2007; Gastal and Gastal, 2011). Ovulation induction allows the timing of ovulation to be coordinated with routine insemination, or when breeding mares with a stallion of low fertility, poor longevity, or with frozen-thawed semen (Norman and Larsen, 2010). Ovulation is routinely induced with human chorionic gonadotropin (hCG), recombinant LH (rLH), or the GnRH analogue Deslorelin. On average, ovulation occurs approximately 36 h after treatment, but the effectiveness of any of these treatments can be affected by the stage of the estrus cycle, follicle size and maturity (Samper, 2008).

In mares, the use of human chorionic gonadotrophin (hCG), which has an LH like action in mares (McCue *et al.*, 2004), or GnRH to induce ovulation can help to further optimize the time of insemination relative to ovulation (Sieme and Klug, 1996). GnRH pulses are followed by LH pulses from the pituitary gland, generally together with a FSH pulse (Aurich, 2011; McKinnon, 2011). The use of GnRH analogue deslorelin (OvuplantTM) in the form of a small implant has been approved for induction of ovulation in mares. OvuplantTM is labelled to be used on mares displaying behavioral estrus and with a follicle of at least 30 mm in diameter (McKinnon *et al.*, 1993).

It has also been hypothesized that an ovulatory dose of hCG induces the cessation of follicle growth there by reducing the follicle diameter during the preovulatory period (Gastal *et al.*, 2006a). A trail with hCG of 2,500-5,000 IU, IV or IM, was administered to hasten ovulation of a dominant follicle during estrus and showed, if the mare has a preovulatory follicle ≥35 mm diameter, ovulation occurs within 36-48h after administration (Patricia, 2018).

3. ULTRASONOGRAPHY OF MARE REPRODUCTIVE ORGANS

An ultrasound is an electronic instrument that sends out ultrasonic sound waves from an attached device called a transducer; a device which is actuated by power from one system to supply power, in any other form, to a second system. Acoustic transducers are used to convert ultrasonic energy to or from electrical, mechanical, or thermal energy. The waves pass freely through fluid and are reflected back to the probe once they contact a soft tissue like muscle or a dense structure like bone, resulting in an image (Walcott and Allen, 2005)

Ultrasonography has brought a powerful dimension to the evaluation of the equine reproductive organs. It is technique for imaging structures to identify, measure and assess physiologic status of soft tissues (Pycock, 2002; Watson *et al.,* 2003). High-resolution ultrasonographic machines with B-mode (gray-scale) and color- and Power-Doppler modes have been used recently in studies of preovulatory follicle characteristics with different goals (Gastal *et al.*, 2006a).

The use of transrectal ultrasonography to evaluate the dynamic changes of the equine uterus has been a major advance in equine reproduction. It is important for diagnosing and monitoring of biologic and pathologic reproductive events in horses, as well as in cattle, in both clinical and research areas (Ginther, 1995). Moreover, the transvaginal route is used for ultrasound-guided entry of specific anatomic targets for recovering or sampling of fluids and tissues (eg, oocyte aspiration, luteal biopsy) and inserting substances (eg, embryos, semen, follicular factors, hormones), as well as twin reduction (Brinsko *et al.,* 2010).

Ultrasonography is a significant contributor to reduction of the number of breeding or inseminations required per estrus (Slusher *et al.*, 2004). Monitoring and determining the stage of the estrus cycle and responses to uterine therapies, are some of the uses of ultrasonography in equine reproduction. Normal mares under the influence of estrogen will have edematous hyperplasia of the endometrial folds. Endometrial edema increases progressively during the first few days of estrus, but decreases as the mare approaches ovulation (Samper, 1997; Lemma et al. 2006b). Samper (1997) found small average value of endometrial folding of 1.3 immediately before ovulation; whereas McKinnon *et al.* (1987) stated zero scoring indicates approaching time of ovulation.

Mares must be monitored when they come into heat for follicular development. Follicles on an ovary contain a single oocyte, or egg, and typically grow 3-5 mm per day but can vary depending on horse breed (Aurich, 2011). Parameters of follicular size, follicular consistency, cervical size and consistency, and uterine tone can be monitored through rectal palpation and ultrasonography (Samper, 1997; Stout, 2003). A mare with a large, very soft follicle that has an open cervix is a prime candidate for breeding (Slusher *et al.*, 2004). During AI, mare should be examined by ultrasound every day or every other day when the ovarian follicle reaches a size of 35 mm and the mare shows strong signs of heat and inseminated every 48 hours until she ovulates (Aurich, 2011; Patricia, 2018).

In mares, early diagnosis of pregnancy can be done due to the spherical shape and large dimensions of the fluid-filled embryonic vesicle when compared to other species. The ultrasonic visualization of conceptus is possible from day 9 of pregnancy, when the embryonic vesicle presents between 1 to 2 mm of diameter, being identified as a spherical shape structure enclosed by an echogenic capsule (Ginther, 1983). Ultrasound examination on or before day 16 is also beneficial for the identification and management of twins, scheduling of rebreeding in open mares, and early detection of problems associated with pregnancy.

4. ARTIFICIAL INSEMINATION IN MARE

Artificial insemination involves the collection of semen from a male, usually of superior genetic merit, followed by the transfer of quality semen into a sexually receptive female at the time of ovulation, in order to result in fertilization. AI may be used to overcome problems that may preclude a mare from natural service, removal of geographical restrictions through semen transportation, permitting the storage of semen for future or assist in the preservation of breed after the death of the stallion. It also allows a significant increase in the number of mares a stallion may cover during one season or through reproductive life, permitting the use of fixed-time AI and potentially reducing labor costs, control of disease and reduce risk of injury to the mare, stallion, and personnel (Davis, 1999; McCue, 2012).

Before real time of AI, sperm collected and evaluated for several indicators of fertility, including total and progressive motility, total sperm number (a combination of volume and concentration), and morphology or shape of the sperm (Jasko *et al.*, 1992). After sperm quality evaluation semen can be used as fresh, cooled, or frozen. In using fresh semen, the mare and stallion are at the same location; semen is collected from the stallion and infused directly into the mare within 2h. For cooled semen, the ejaculate is often centrifuged to remove the seminal plasma, divided into several samples, and a substance called "extender" is added to provide a nutrient source and buffer from the elements. It should be cooled to 4-5^{0}C and then packed into specially-designed containers containing insulation and freezer jars for protection and to maintain proper temperature for up to 24h (Jasko *et al.*,1992; Vidament *et al.*, 1997).

Use of frozen semen is becoming increasingly common among breeders and has been frozen in liquid nitrogen at a temperature of -196°C, which stops all motility. By using extender with cryoprotectant to protect cell membranes from the freezing process, semen may be utilized to impregnate mares even after a stallion's injury or death (Samper *et al.*, 2002). Frozen semen is stored in small 0.5ml plastic "straws," which allows for semen from a single collection to be divided into many doses. Immediately prior to insemination, straws are thawed in a warm-water bath at 37°C for 30 seconds, with exact precision required in temperature and timing to maximize sperm survival (Samper *et al.*, 2002).

Time of ovulation and AI is critical which is important to inseminate the mare between 0 and 48 hours prior to ovulation to offer a good chance of fertilization and to prevent multiple inseminations per cycle that increases the risk of endometritis, costs of services and semen (Samper, 1997; Gastal and Gastal, 2011). To maximize pregnancy rates, natural mating should be within 48 h before ovulation; AI with cooled shipped semen, 12-24 h before ovulation (Samper, 2008); and AI with frozen-thawed semen, <12 h before ovulation with accurate prediction of ovulation (Newcombe *et al.,* 2011). Mares bred at 0 to 6 h with frozen semen after ovulation had normal pregnancy rates and did not experience increased embryonic death rates. However, mares bred 6 to 12 h after ovulation had normal pregnancy rates, and experienced an embryonic loss. Presumably, aging gametes can adversely affect developmental competence of the embryo. A good fertility has been reported when mares were bred after ovulation, but a breeding strategy of post-ovulation breeding with fresh semen is not generally recommended (Brinsko *et al.*, 2010).

The rate of pregnancy in Arab pure bred mares were varied significantly according to the moment of AI (21.5% if the AI occurs at 4pm versus 36% and 36.5% respectively if the AI is practiced immediately after the 9am and 11pm after ultrasound examinations and inseminated intra-cornually in post-ovulation (Najjar *et al.*, 2018). The effects of pre-ovulatory and post ovulatory insemination on pregnancy rate were assessed by Woods *et al.* (1990) between different experimental group of animals. Pregnancy rate within the pre-ovulatory group was lower (P<0.05) for insemination 4 days or more before ovulation than for up to 3 days before ovulation. Pregnancy rate was greater (P<0.05) for mares inseminated 0 to 6 h after ovulation than for mares inseminated at 18 to 24 h and no pregnancies occurred when mares were inseminated 30 h or more after ovulation.

5. FACTORS AFFECTING CONCEPTION RATE TO FROZEN SEMEN IN MARES

Growth of antral follicles in the ovary occurs in wave-like patterns, and is influenced by several factors such as stage of the estrus cycle, season, pregnancy, age, breed and the individual (Dolezel *et al.*, 2012; Raz and Aharonson-Raz, 2012).

The effect of breed on conception rate to AI were determined in different research work of the world. The pregnancy rate of Criollo breed (56.2%) and Thoroughbred mares (53.7%) under AI was reported by Camargo *et al.* (2017) and Sharma *et al.* (2010) respectively. A 67.8 %, 45% and 69.6 % pregnancy rate in the study of Rota *et al.* (2004), Squires *et al.* (2006) and Crowe *et al.* (2008) respectively were also reported in different breeds of mares. Numerous factors influence the pregnancy rate in horses bred by AI, which includes the inherent fertility of the mare and stallion, the type of semen used for insemination (Jasko *et al.,* 1992), the number of spermatozoa in the insemination dose (Vidament *et al.,* 1997; Pickett *et al.,* 2000), number of services per conception (NSPC), timing of AI relative to ovulation, season of study, difference in animal management and other animal related factors.

Some researchers found that, reproductive efficiency declines with increasing age in the mare as indicated for decreased pregnancy rates and increased embryo-loss rates in older mares (Morris and Allen, 2002; Hemberg *et al.,* 2004). Conversely, other researchers found that age has no significant effect on fertility (Sieme *et al.,* 2003; Rossi *et al.,* 2014). Vidament *et al.* (1997) reported that pregnancy rates were found to be higher in <16 years old mares than ≥16 years old mares with frozen-thawed semen. Similarly, a pregnancy rates in 4-11 years old mares were found to be higher than 12-19 years old mares as described by Mustafa *et al.* (2016) in Arabian mares and indicated a rate of 31.3 ± 11.7 and 28.1 ± 8.2 % respectively. The decline in fertility with age may be attributed to age related changes in the uterus, changes in hormonal and follicular wave characteristics or defective oocytes (Ginther, 1993; Davies Morel *et al.,* 2010). Older mares, if mated towards the end of the breeding season and have more than one large follicle, are significantly more likely to have smaller follicles. Follicle size is related to reproductive success; as small pre-ovulatory follicle size may account, for the poorer reproductive performance (Shirazi *et al.*, 2002).

Mares in body conditions of four or less will be poor breeders and more susceptible to pregnancy losses than mares maintained at higher body condition scores. Body condition of four or less will delay the time of their first ovulation of the breeding season and expected to require more cycles per conception. They requiring an average of three cycles before settling, as compared with one and one-half cycles per conception for similarly managed mares with condition scores of five or higher (Freeman, 2014).

The dose of semen is influential for pregnancy success in mares. The minimum recommended number of spermatozoa included within a conventional insemination dose is generally $300×10^6$ progressively motile spermatozoa for fresh (Vidament *et al.,* 1997; Gahne *et al.,* 1997) and $500×10^6$ progressively motile spermatozoa for frozen semen (Gahne *et al.,* 1997). In the study of Mustafa *et al.* (2016), the lowest and highest pregnancy rates were obtained for $400×10^6$ and $1200×10^6$ doses respectively. Pickett *et al.* (2000) recommend for each insemination dose 500 $x10^6$ fresh progressively motile sperm at the stud farm, 1000 $x10^6$ progressively motile sperm for shipped semen at the time when the semen is packed before cooling, and 800 x 10^6 total frozen sperm with at least 30% post-thaw progressive motility for maximum reproductive efficiency.

On the other hand, the pregnancy rates in mares inseminated before ovulation were found to be higher than those inseminated after ovulation. A group of randomly inseminated Arabian mares before (when follicle diameter was 40-45mm) and after ovulation showed respectively an average pregnancy rates of 37.5 ± 9.6 and 20.8 ± 9.7 % in the study of Mustafa *et al.* (2016).

Pregnancy rates per cycle was also reported as 77 % for fresh semen in retrospective study of Squires *et al.* (2006). Relatively poor pregnancy rates after AI with frozen-thawed semen as compared to fresh or chilled stallion semen are common (Jasko *et al.*, 1992; Vidament *et al.,* 1997), and are generally accepted to result from damage suffered by sperm during the freezing and thawing process. Sieme *et al.* (2003) found that in relation to insemination with frozen semen, a single insemination should preferably be carried out between 12 h before and 12 h after ovulation. On average, the number of cycles per pregnancy are increased and the pregnancy rate per cycle is decreased when mares are bred with frozen-thawed semen compared to natural cover or fresh semen artificial insemination (Squires *et al.,* 1999). Increasing the number of cycles that a mare is bred will increase the chances of particular mare becoming pregnant at the end of the season (Samper *et al.*, 1991).

Sperm cells can be observed 30 minutes after artificial insemination and remain in the uterine tubes for at least 24 h. At this moment there is an inflammatory reaction (acute endometritis), which is considered as physiological and aims to remove excess spermatozoa, seminal plasma and contaminants prior to entry of the embryo in the uterus (Troedsson, 1997). The sperm are removed from the female genital tract by phagocytosis or by physical cleaning. But, the sperm distribution and its function are influenced by local deposition of semen, the semen characteristics, the anatomy of the female genital tract, myometrial contractility and the microenvironment of the lumen which can in turn affect pregnancy success (Fiala, 2012).

6. MATERIALS AND METHODS

6.1. Study area

The study was carried out in Addis Ababa at Balderas sport horses and recreation directorate of Palace administration. The center lasts for nearly one century with primary objectives of some horse sports. Recently horse reproduction for breed improvement and preservations are main activities working in the center in addition to sports and recreational services. The area located at about 12km from the Palace Administration at 9°N latitude and 38°E longitude with 2400 meters above sea level. Addis Ababa has an annual rainfall of 1800 ml which falls during the long rainy season extending from June to September and short rain fail extending from March to May. The mean annual maximum and minimum temperature ranges are 23°C and 10.7°C respectively.

6.2. Study animals and management

The study animals were local breed of Selale type mares as characterized by Kefena *et al.* (2012) and exotic cross bred (Anglo X Arabian) mares which are kept under sport horses and recreational directorate of the palace administration. Currently, horses in the center are used as horse racing, renting for recreational purpose and cart pulling, involved in exhibition and historical reenactment or national ceremony. Animal management includes regular deworming, Brucellosis screening test (RBPT), and vaccination against African horse sickness (AHS). Wheat bran and good quality hay with mineral and vitamin supplements are commonly supplied feed type for individual animals in separate pen based on body weight, production level and service provision. Water is provided *ad libitum*. Animals are kept in moderate house with adequate space which were cleaned 3-4 times/day.

During selection of study animals, clinical and ultrasonic examinations of reproductive organs were done to assess any abnormalities that can affect reproductive performances of mares. A total of 23 mares (11 local and 12 crossbred) with no ovarian or uterine abnormalities were included in the study.

6.3. Study design

Experimental study was carried out from January to June, 2018, to determine ovarian follicular dynamics and conception rate to AI with frozen semen in mares. After selection, the study animals were followed for 2 consecutive estrus cycles before AI was started. During each estrus cycle transrectal ultrasonographic examinations was carried out to measure ovarian follicular growth and other developmental changes on the uterus. In the following cycle, the mares were allocated for estrus induction using single $PGF_{2\alpha}$ injection and followed for AI until pregnancy was achieved. Teasing scores were estimated and recorded based on the criteria set in (Annex-I) when animals were in estrus phase, to correlate with follicular size and relative time of ovulation. Individual animal's data such as, breed, age and body condition scores (BCS) were also collected to assess the effect of breed on follicular growth pattern and other animal related factors on conception rate to AI. BCS was determined on 1-9 scale according to Henneke *et al.* (1983) and further classified into three groups as (<4, 4-6 and >6 scores).

6.3.1. Ultrasonographic evaluation of reproductive organs

Daily ultrasonic examinations of reproductive organs were done by scanning of the mare's uterus, and ovarian structures for 2 consecutive estrus cycles as described in (Ginther, 1995), using Aloka B-mode, real time ultrasound scanner with a 5 MHz linear array transducer (Aloka 500, S. Korea). Uterine cross sectional diameter, number of follicle in each ovary and the diameter of the first three largest follicles were measured during each ultrasound scan using the internal electronic caliper. Ovulation was determined by the disappearance of a largest follicle observed during a previous examination and its replacement by a corpus haemorrhagicum or CL. Inter ovulatory interval was defined as the period from one ovulation to the preceding ovulation as confirmed by ultrasonography. Development of endometrial fold for individual animals were scored through the cycle to see its relationships with size of developing largest follicle and uterine diameter. Ultrasonic endometrial echotexture was scored on a scale of 0-4 based on the degree of endometrial folding (zero for invisible folding; four as maximal). Evaluated data provide enough information on the number of follicles, the maximum diameter of the dominant follicles, uterine diameter, the growth rates of the dominant follicles and the lengths of IOI.

Growth rate of dominant follicle was determined by dividing the difference of maximum and minimum diameter of the dominant follicle (when follicle size was ≥20mm) for duration of growth as defined by Gastal *et al.* (1997).

6.3.2. Artificial insemination and pregnancy diagnosis

Estrus was synchronized at the beginning of the study using a single 2ml IM injection of $PGF_{2\alpha}$ (A 500µg Cloprostenol, Bremer Pharma Warburg, Germany) based on the detection of active CL. Mares that did not have active CL did not receive the treatment. All mares were then monitored by transrectal ultrasonography to determine ovarian follicular activity. Mares were subjected to measurement of uterine diameter, score of endometrial fold, teasing, and measurement of the diameter of the preovulatory follicle to estimate time of ovulation. Ovulation was induced using an intramuscular injection of 2ml Receptal (40µg Buserelin acetat, Intervet International, Germany) when the preovulatory follicle reached 40mm and ovulation did not occur. AI were performed post ovulation using imported Hanover breed semen (Landgestuet Celle, Germany) with dose rate of $400x10^6$ sperm/dose for each insemination. Inseminations were done within a range of 6-9 h of an ultrasonic confirmation of ovulation. The AI procedure was strictly aseptic, intravaginal deposition of the semen in the uterine body (or horns when time was longer after ovulation) using equine insemination catheter.

Pregnancy was evaluated ultrasonically at 2-3 weeks later post AI. The ultrasound examination was carefully performed by imaging the whole uterus. A mare was diagnosed as pregnant when an embryonic vesicle was identified within the uterus.

6.3.3. Data management and analysis

All collected data were managed in Microsoft excel sheet. Using STATA version 12 (1985-2011 Stata Corp LP, College Station, Texas 77845 USA), all data were described using a descriptive statistic; whereas independent sample t-test and one-way ANOVA were used for analysis of follicular data, uterine diameter, IOI, phases of estrus cycle to compare the means between breeds.

Pearson's correlation was used to evaluate the presence of correlations between uterine diameter, teasing score and development of endometrial fold with size of the developing dominant follicle as well as growth rate of dominant follicles with IOI. Pearson's correlation coefficient (r) was used to see strength of relationship between the measured parameters by taking extreme values, either +1 or -1. Chi square test and Fisher's exact test were used to see the effect of animal related factors on conception rate to AI with frozen semen in mares. For all data analysis the significance value was set at $P<0.05$.

6.4. Ethical considerations

Ethical clearance for this study was obtained from animal research ethical review committee of Addis Ababa University, College of Veterinary Medicine and Agriculture, with reference number of approval letter VM/ERC/15/05/10/2018 (Annex-V).

7. RESULTS

7.1. Ultrasonic follicular data

Daily scanning of the mare's uterus, and ovarian structures performed using rectal ultrasonography for two consecutive estrus cycles were presented as mean (±SEM) (Table 2). The mean (±SEM) of cross sectional uterine diameter between local and cross breeds were similar; whereas endometrial fold development through estrus cycle indicated significant difference at $P<0.05$. Concerning the number of follicles, cross breed had significantly higher number of follicles than local breed mares. Overall mean (±SEM) of the number of follicles were 17.4±0.8 in cross breed and 13.2±0.4 in local breed mares.

Table 2: Results of ultrasonic evaluation of the reproductive organs between breeds of mares

Ultrasonic measurement	Local breed			Cross breed		
	N	Mean ±SEM	Range	N	Mean ±SEM	Range
Uterus						
Uterine diameter (mm)[b]	152	44.4±0.5	20.2-56	156	45.0±0.5	27.1-58.4
Endometrial fold score [a]	141	3.1±0.1	0-4	136	2.8±0.1	0-4
Right Ovary (ROV)						
Number of follicles [a]	94	6.9±0.3	3-16	72	8.7±0.6	2-17
1st largest Follicles(mm)[b]	184	28.2±0.8	5.2-54.4	163	28.9±0.9	10.9-57
2nd largest Follicles(mm)[a]	182	17.8±0.5	4.2-38.1	161	19.4±0.5	6.4-35.7
3rd largest Follicles(mm)[a]	180	13.7±0.4	3.2-29.3	160	15.3±0.4	5.5-30
Left Ovary (LOV)						
Number of follicles [a]	94	6.3±0.2	3-14	72	8.6±0.4	3-16
1st largest Follicles(mm)[b]	188	29.4±0.9	8.7-58.1	165	28.6±0.8	9.2-55.7
2nd largest Follicles(mm)[a]	181	18.5±0.5	5.7-33.4	162	21±0.6	5.4-49.1
3rd largest Follicles(mm)[a]	178	14.3±0.4	4.1-28.2	162	16.4±0.5	4.9-36.1
Total number of follicles [a]	94	13.2±0.4	6-24	72	17.4±0.8	3-30

N: number of measurements, SEM: Standard error of the mean, Superscript a represent $P<0.05$ and b for $P>0.05$ for mean difference between breeds

Collected data were interpreted for wave of follicular development and characteristics within IOI for the study animals. The study result indicated, only one wave of follicular development per estrus cycle in which all of the developing dominant follicles were ovulated without regressing. In the two consecutive estrus cycles of the study, double ovulation recorded only for one animal. The mean (+SEM) of preovulatory follicle diameter in local and cross breed mares indicated 49.1+1.0mm and 50.1+0.8mm respectively. The higher IOI in local (24.3+0.4 days) than cross breed mares (22.7+1.0 days) where resulted from faster growth rate of dominant follicles in cross than local breed mares. Duration of estrus cycle indicated closely similar between both breeds with slightly longer estrus and diestrus phases in local than cross breed mares. Length of estrus phase was 7.0+0.9 and 6.1+0.6 days for local and cross breeds respectively (Table 3).

Table 3: Diameter of preovulatory follicles and characteristics within interovulatory interval between local and cross breed mares

	Local breed			**Cross breed**		
Measurement	**N**	**Mean + SEM**	**Range**	**N**	**Mean + SEM**	**Range**
Diameter of Pre-ovulatory follicle (mm)	22	49.1+1.0	38-55.6	22	50.1+0.8	42.7-57
Growth rate of dominant follicle (mm/day)	21	2.8+0.1	1.4-3.8	18	2.9+0.2	1.2-4.1
IOI (days)	9	24.3+0.4	22-26	10	22.7+1.0	18-27
Length of estrus (days)	9	7.0+0.9	3-13	10	6.1+0.6	4-10
Length diestrus (days)	9	17.1+1.0	12-20	10	16.9+0.6	14-22

N: number of measurement, SEM: Standard error of the mean

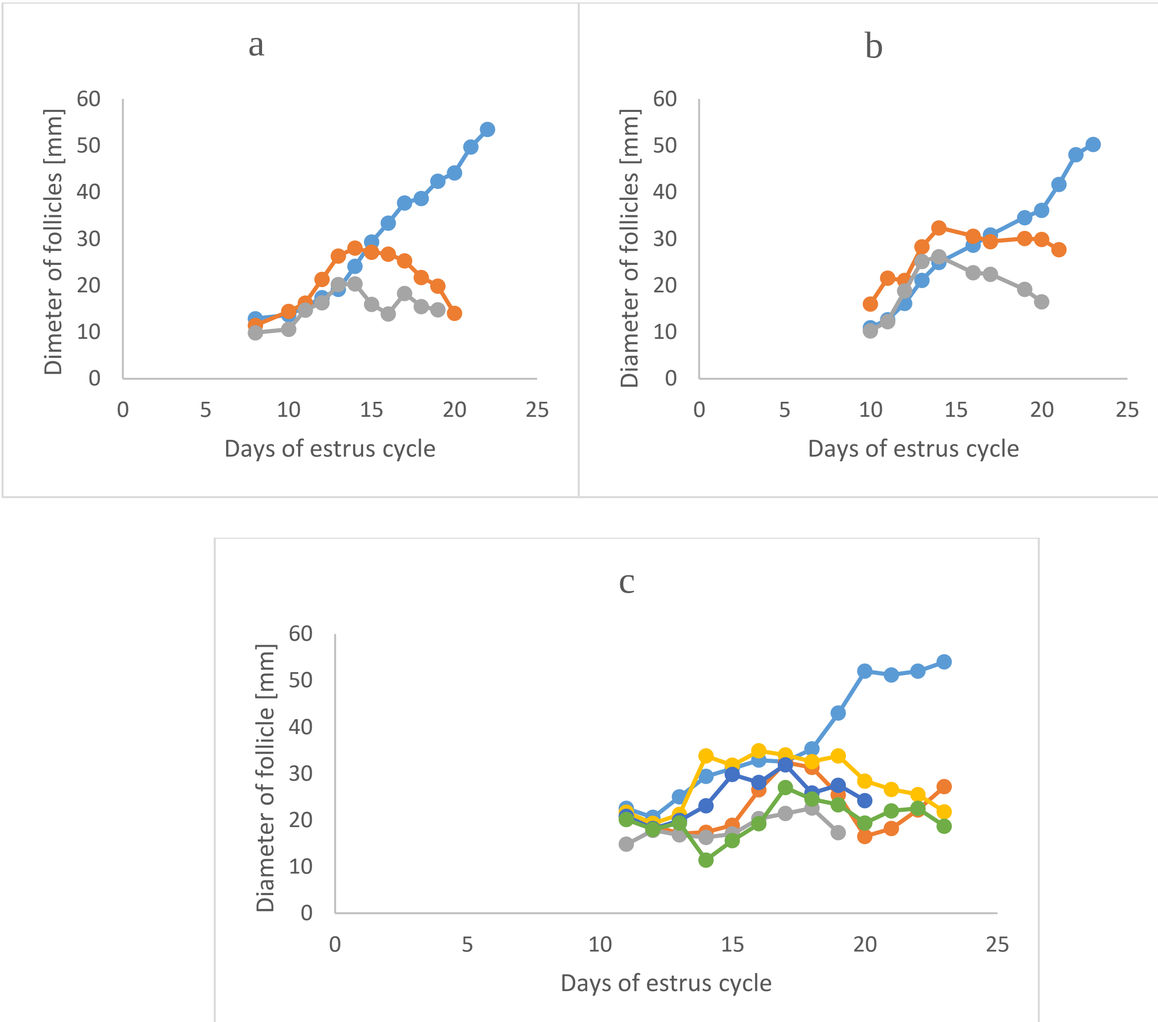


Figure 2: Graphical representation of estrus cycle and diameter of developing follicles in local and cross breed mares: a) Average diameter of first three largest developing follicles in cross breed mares b) Average diameter of first three largest developing follicles in local breed mares c) Representation of one wave and single ovulation of developing follicles in estrus cycle of individual mare.

In the present study data between two consecutive estrus cycles were compared to characterize changes on uterine and ovarian structures at late luteal and estrus phases of the cycles. A cross sectional uterine diameter, endometrial fold score and number of follicles showed significantly higher measurements in the second cycle than first estrus cycle. similarly, the diameter of preovulatory follicles were higher in the second cycle (50.9±0.9mm) than first cycle (48.1±0.9mm) with statistically significant difference at $P<0.05$ (Table 4).

Table 4: Mean uterine diameter and ovarian follicles between consecutive estrus cycle

Ultrasonic measurement	**First cycle**			**Second cycle**		
	N	**Mean + SEM**	**Range**	**N**	**Mean + SEM**	**Range**
Uterine diameter (mm)[a]	106	43.6+0.6	20.2-56	65	48.6+0.4	33.9-54.6
Endometrial fold score [a]	97	2.9+0.1	0-4	65	3.4+0.1	2-4
Diameter of Preovulatory follicles(mm)[a]	21	48.1+0.9	42.7-58.1	20	50.9+0.9	38-55
Diameter of 1st largest follicles on ROV (mm)[b]	138	32.4+0.8	20-57	99	34.3+1.1	20.4-55.1
Diameter of 1st largest follicles on LOV (mm)[b]	134	34.9+0.9	21.2-58.1	93	33.8+1.0	21.2-55
Number of follicles on ROV [b]	45	7+0.6	2-16	62	7.7+0.5	3-16
Number of follicles on LOV [a]	42	5.7+0.4	3-12	62	7.6+0.5	4-16
Total number of Follicles [a]	44	13.1+0.9	6-27	62	15.5+0.7	6-27

N: number of measurements, SEM: Standard error of the mean, Superscript a represent $P<0.05$ and b for $P>0.05$ for mean difference between estrus cycle

Relationships among variables were described using Pearson's correlation and showed a positive correlation between diameter of largest follicles with uterine diameter (r=0.5), diameter of largest follicles and endometrial fold (r=0.56), diameter of largest follicles and teasing score (r=0.69) and diameter of largest follicles and IOI (r=0.19). However, a negative correlation between the growth rate of dominant follicles with IOI (r= -0.17) was seen.

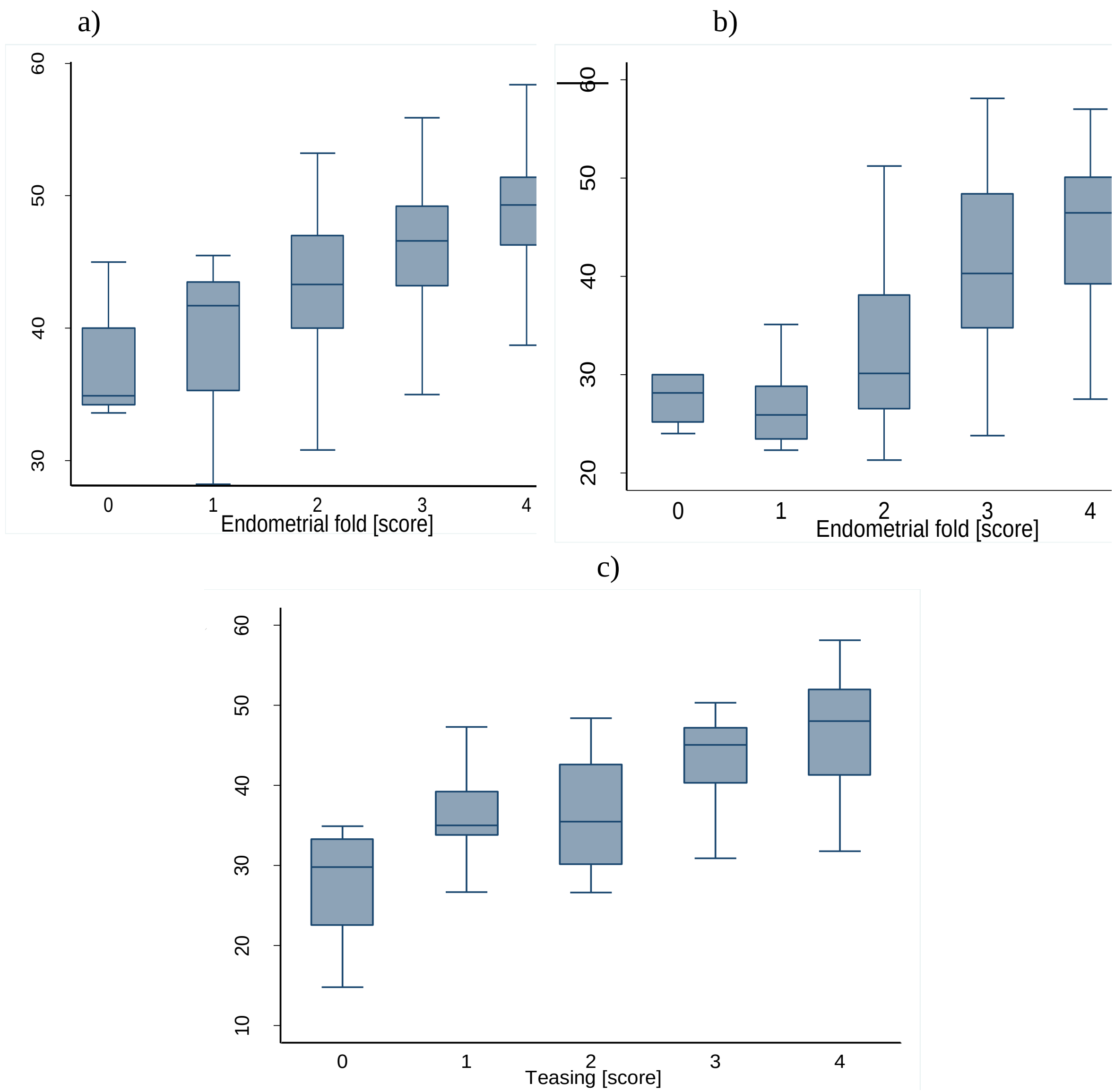


Figure 3: Graph showing correlation of teasing score, uterine diameter and endometrial fold score with diameter of developing dominant follicle as (Mean +SD): a) Correlation between mean (+SD) of endometrial fold score with uterine diameter b) Correlation between mean (+SD) of follicle diameter with endometrial fold score c) Correlation between mean (+SD) of follicle diameter with teasing score

7.2. Conception rate to AI with frozen semen

The overall conception rate to AI with frozen semen in the present study was 15/21 (71.43%) with no significant differences between breed of mares (P>0.05). Age and BCS of animals were also non significantly affect conception rate to AI (Table 5).

Table 5: Effect of breed and other animal related factors on conception rate to frozen semen

Factors	N	Positive	Percent (%)	Fisher's exact
Breed				
Local	10	7	70.0	
Cross	11	8	72.73	0.633
Total	21	15	71.43	
Age (years)				
≤10	15	10	66.67	
>10	6	5	83.33	0.424
Total	21	15	71.43	
BCS				
≤4	9	7	77.78	
4-6	6	4	66.67	1.00
>6	6	4	66.67	
Total	21	15	71.43	

N: number of animals

In the present study side of the ovary have no significant effect on conception rate to AI with frozen semen. However, a slightly higher conception rate was obtained when ovulation was from right ovary than when ovulated from left ovary. Conception rate to AI was significantly affected by number of service per conception P<0.05, (i.e conception rate increased with increased number of services/conception) (Table 6).

Table 6: Effect of the side of ovulatory follicles and number of services/conception on conception rate to frozen semen

Factors	N	Positive	Percent (%)	χ^2	P-value
Ovary with ovulatory follicles					
ROV	24	9	37.50		
LOV	23	6	26.09	0.7040	0.401
Total	47	15	31.91		
NSPC					
One	21	3	14.29		
Two	17	6	35.29	8.0950	0.017
Three	9	6	66.67		
Total	47	15	31.91		

N=number of measurements, NSPC= number of services per conception

The overall mean (±SEM) number of service (cycle) per conception was 2.2±0.2, with cross breed having 1.8±0.3 and local breed having 2.7±0.2 services/conception with statistically significant difference between breed of mares (P=0.005). The higher NSPC for lower BCS than higher BCS were reported in the present study with mean (±SEM) of 2.7±0.2, 1.8± 0.3, 1.8±0.4 for body condition of ≤4, 4-6 and >6 scores respectively at P>0.05.

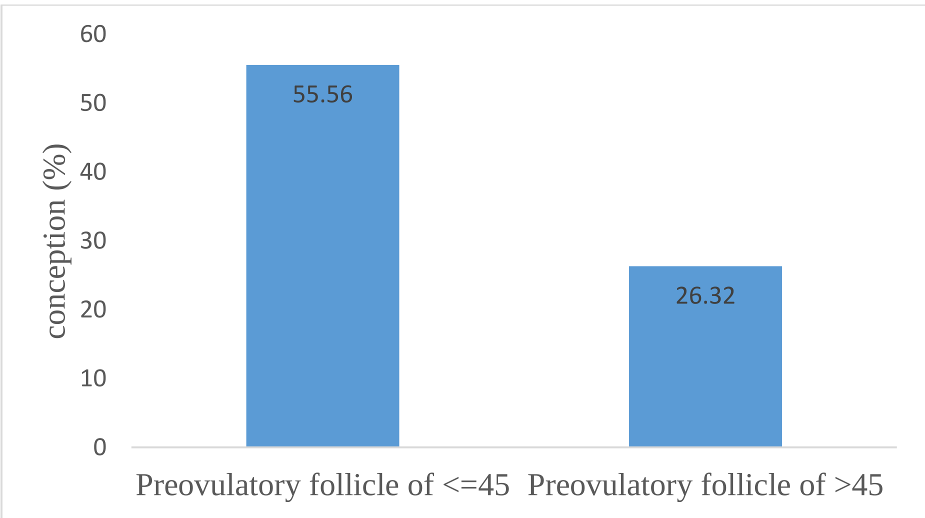


Figure 4: Variation of conception rate in mares based on diameter of preovulatory follicles

8. DISCUSSION

This research was found different aspects of mare's uterine and ovarian follicular dynamics including uterine diameter, endometrial fold development, ovarian follicular size, number of follicles in each ovary and growth rate of dominant follicles, phases of estrus cycle and IOI from routinely collected data of transrectal ultrasonography. Conception rate to AI with frozen semen post ovulation and effect of breed and other animal related factors were determined.

In this study the mean (±SEM) of cross sectional uterine diameter between local and cross breed mares were 44.4±0.5mm and 45±0.5mm respectively with no significant difference. Previous studies of Lemma *et al.* (2015) and Griffin and Ginther (1991) reported mean uterine diameter of 35.1±5.8mm and 30.1-31.7mm respectively. In study of Griffin and Ginther (1991) uterine dimeter was increased significantly between 11 and 21 days of estrus cycle. This implies larger diameter at end of the cycle indicate relative time of ovulation for reproductive efficiency. The difference in diameter will be affected by season of the study, difference in breed or technical error of measurement. Higher uterine diameter in the present study will be due to delayed time of ovulation after the follicle size reach the standard (≥ 35mm) which will cause continues growth of uterine and ovarian structures.

A significant mean differences of endometrial fold scores were found between breeds as 3.1±0.1 in local and 2.8±0.1 scores in cross breed mares at $P<0.05$. In contrast to this, Samper (1997) found small average value of endometrial folding of 1.3 immediately before ovulation; as well as McKinnon *et al.* (1987) stated that a reduction in endometrial folding to zero indicates approaching time of ovulation. Even level of the scores different between breeds the present result was agreed with the scientific fact of endometrial folding in mares correlates with estrogen production in the dominant follicle and usually starts to be noticeable on day seven before ovulation; then maximum near to time of ovulation and subsequently diminishes and disappears after ovulation (Pycock, 2002).

In each ovary the mean diameter of 1^{st} largest follicle compared between breeds and resulted in closely similar diameter. The mean (±SEM) on both ovaries ranges from 28.2±0.8mm to 29.4±0.9mm with non-significant differences between breeds. The study of Lemma *et al.* (2015)

reported mean diameter of 19.6±6.9mm to 22.0±9.4mm in range on both ovaries. The mean diameter of 2nd and 3rd largest follicle showed significant difference (P<0.05) between breeds with higher diameter in cross breed in respective to each ovary. The variation will be due to difference in level of plasma circulating FSH and other inhibitory factors between breeds that affect size of subordinate follicles (Bergfelt *et al.,* 2001).

The number of counted follicles in each side of ovary had a significant difference (P<0.05) between breeds with frequently higher number counted in cross breed than local breed mares. There is also slightly higher number of follicles on right ovary than left ovary. The overall mean (±SEM) in number of follicles were 17.4±0.8 in cross breed and 13.2±0.4 in local breed mares. Similar result was reported by Lemma *et al.* (2015) for cross breeds with 16. 2±6.l total number of follicles with in the same study area. Approximately 7-11 follicles per wave emerge at diameters of 5-6 mm that enter a common-growth phase of about 6 days as reported by Gastal *et al.* (1997) was similar with average number of follicles on one side of ovary in the present study. Higher number of follicles in cross breeds of mares might be one indicator of the differences in fertility level between breeds.

Diameter of preovulatory follicle in local and cross breed of present study indicated mean (±SEM) of 49.1±1.0mm and 50.1±0.8mm respectively. This is comparable with the research work of Dolezel *et al.* (2012), who reported the diameter of 48mm in Czech warmblood mares, Ginther and Pierson (1989) with preovulatory follicle size of 46 mm and 48 mm at different season of study. Other researchers like (Dimmick *et al.,* 1993; Shirazi *et al.,* 2002; Ginther *et al.,* 2004; Ginther *et al.,* 2008c; Camargo *et al.,* 2017) reported lower preovulatory follicle diameter of 40-45mm than the present study on the day before ovulation. The difference in diameter of preovulatory follicles might be affected by breed, season of the study (Ginther and Pierson,1989; Dolezel *et al.,* 2012), number of ovulation as single or double ovulation per cycle (Ginther *et al.,* 2008c), as well as age of animals as confirmed by Davies Morel *et al.* (2010). Cross breeds of the present study animals have higher body size then local breeds with relatively the same diameter of preovulatory follicles; from this, the present result disagree with the work of Dimmick *et al.* (1993) who concluded that, the smaller follicle size at ovulation may be related to body size of mares.

Growth rate of dominant follicles were slightly higher in cross breed (2.9±0.2mm/day) than local breed mares (2.8+0.1mm/day); this resulted in higher IOI in local (24.3±0.4 days) than cross breed mares (22.7±1.0 days). This result was agreed with the experimental work of Gastal *et al.* (2004), Aurich (2011) and Dolezel *et al.* (2012) who found growth rate of 2.8mm/day, 3mm/day and 2.8mm/day respectively. A 5.2mm/day growth rate in hCG treated experimental group of animals by Dolezel *et al.* (2012) and 3.4±0.2mm/day in induced ponies' mares (Ginther *et al.,* 2008d) were higher than growth rate of the present study. Rate of follicle growth can be influenced by stages of estrus cycle at which the diameter of follicles measured, as well as induction of ovulation and estrus in addition to breed differences.

The mean length of IOI in present study was closely similar with the findings of Griffin and Ginther (1991) and Shirazi *et al.* (2002) who reported an interval of 23 and 22.1± 0.43 days respectively, but higher than 19.57 ± 1.8 days (Ali *et al.,* 2014). A minor difference between the study will be due to the effect of breed, age of study animals as growth rate of the dominant follicle lower and IOI may be longer in old mares than young and middle-aged mares (Ginther *et al.,* 2008b). Induction of ovulation decreases the size of the preovulatory follicles in comparison with natural ovulation and this shorten again the IOI (Gastal *et al.,* 2006b).

The length of estrus cycle phases indicated close similarity between both breeds with slightly longer estrus and diestrus phases in local than cross breed mares. Estrus phases were 7.0±0.9 and 6.1±0.6 days for local and cross breeds respectively; whereas the mean diaestrus phase were 17.1±1.0 days for local and 16.9±0.6 days for cross breed mares. The present result was consistent with the report of 5-7 days of estrus with cycle length of 22 days (Aurich, 2011), average estrus duration of 6.58 ± 1 days (Ali *et al.,* 2014); but higher than diestrus period of 14 to 15 days (Ginther, 1992; Raz and Aharonson-Raz, 2012). An estrus length of 4.9±0.38 days in quarter horse mares (Dimmick *et al.,* 1993) was lower than the present findings. The differences might be due to functional differences of ovary between breed of mares. In addition to this, season affects the duration of follicular phase in which the duration of estrus decreases during the summer, which most likely represents an acceleration of folliculogenesis before ovulation (Ginther, 2000).

Ovarian follicles and uterine structures were characterized between two consecutive estrus cycles in the present study. Accordingly, a cross sectional uterine diameter of 48.6±0.4mm in the second cycle were higher than 43.6±0.6mm of the first cycle; similarly, endometrial fold score of 3.4±0.1 in second cycle were significantly higher than 2.9±0.1 of the first cycle at $P<0.05$. Even the diameter of first largest follicle in left and right ovary were closely similar between the two cycles, the diameter of preovulatory follicles were higher in the second cycle (50.9±0.9mm) than first cycle (48.1±0.9mm) with statistically significant difference. The total number of follicles in both ovaries resulted in mean (±SEM) of 15.4±0.4 for second cycle and 13.1±0.9 for first cycle with significant difference. The variation in activities of ovarian function from one cycle to another in the present findings, related to justification in the study of Lemma *et al.* (2006a) who stated follicle size and number would be affected by feed availability and body conditions of the study animals from one season to another.

Pearson's correlation used to correlate different parameters during estrus cycle of the mares to see their relationships with developing dominant follicles. The study revealed a positive correlation of cross sectional uterine diameter and endometrial fold scores with diameter of developing dominant follicles. A similar finding was reported by Lemma *et al.* (2006b) who found strong positive correlation of indicated parameters in tropical jennies (Equus asinus). In the study of Griffin and Ginther (1991) a positive relationship between changes in uterine diameter and endometrial echotexture ($r = 0.75$) was seen, as the same result was demonstrated in present study (Figure 3a). This study also proves the previous description of, follicle size has been a good parameter to determine the timing of breeding in horses (Samper, 2009) and endometrial fold scores were suggested as a reliable predictor of imminent ovulation (Pycock, 2002).

Teasing scores showed strong correlation with diameter of developing dominant follicles (Figure 3c; $r=0.69$). Mares have different level of receptivity to stallion through estrus period for which most managers use teasing in estrus detection for breeding determination or AI in addition to rectal palpation and ultrasonography (Samper, 2008). As maximum diameter of preovulatory follicles are an indicative of a relative time of ovulation (Samper, 2009); a strong correlation with teasing scores in the present study confirms, implementation of teasing in combination with rectal palpation and ultrasonography is best method to determine breeding time and cost effective if used alone with good experience.

Growth rate of dominant follicles have inverse relationship with IOI (i.e. This implies faster growth of the dominant follicle shorten IOI and the vice versa). However, growth rate and days of IOI can be affected by age (Ginther *et al.,* 2008b), stage of estrus cycle and induction of ovulation (Gastal *et al.,* 2006b).

The overall conception rate of 15/21(71.43%) in present study was comparable with previous report of 67.8 % and 69.6 % in the study of Rota *et al.* (2004) and Crowe *et al.* (2008) respectively. However, the present result was higher than rate in group of Arab bred mares hormonally treated with Chorulon® (37%) and the control group (34%) (Najjar *et al.,* 2018). The pregnancy rate of Criollo breed (56.2%) and Thoroughbred mares (53.7%) under AI as reported by Camargo *et al.* (2017) and Sharma *et al.* (2010) respectively were also lower than overall or breed level pregnancy rate resulted in the present study. Pregnancy rates reported as 77 % for fresh semen in retrospective study of Squires *et al.* (2006) was relatively similar with rate of 8/11 (72.73%) for cross breed in this study. Numerous factors influence the pregnancy rate in horses bred by AI, which includes the inherent fertility of the mare and stallion, the type of semen used for insemination (Jasko *et al.*, 1992), the number of spermatozoa in the insemination dose (Vidament *et al.,* 1997; Pickett *et al.*, 2000), NSPC, timing of AI relative to ovulation, season of study, difference in animal management and other animal related factors.

Age of animals in the present study didn't have significant effect on conception rate to AI when age categorized as ≤10 years and >10 years. Similar findings were reported by different scholars (Sieme *et al.,* 2003; Mata *et al*., 2013; Rossi *et al.,* 2014) and support the present result. Exposure to number of services with aging in mares will experience them for inflammatory response and conception to AI which will compensate with high level of fertility in young than older animals in normal cases. Other researchers indicated that pregnancy rates in 4-11 years old mares were found to be higher than 12-19 years old mares as described by Mustafa *et al.* (2016) in Arabian breed mares. Hemberg *et al.* (2004) also reported the effect of age on conception rate as risk of embryonic loss increase with increasing age. When fertility decline with age the authors related to the fact of older mares, are significantly more likely to ovulate from smaller follicles that related in decreased reproductive efficiencies (Shirazi *et al.,* 2002). Similarly, a decline in fertility with age may be attributed to age related changes in the uterus, changes in hormonal and follicular wave characteristics or defective oocytes (Ginther, 1993; Davies Morel *et al.,* 2010).

Body condition of study animals didn't affect significantly conception rate to AI with frozen semen in the present study. Lower body condition animals have reduced ovulatory follicle size (Lemma *et al.,* 2006a); this is one of factor for reduced pregnancy success as larger diameter of follicles expected for higher fertility (Lucy, 2007). Mares in body conditions of four or less will be poor breeders and more susceptible to pregnancy losses and are expected to require more cycles per conception. They requiring an average of three cycles before settling, as compared with one and one-half cycles per conception than condition scores of five or higher (Freeman, 2014). The NSPC and sample size used in the present study will influence the difference of conception rate between body condition scores, as mares with body condition score $\leq$4 required more service per conception than above body condition scores.

A closely similar rate of ovulation when ovulatory follicles were from right and left ovary were seen with slightly higher conception rate of 9/24 (37.5%) when ovulation was from right ovary than 6/23 (26.09% when ovulated from left ovary. In opposite to this, there is significantly more pregnancies if the ovulation occurs in the right ovary (59%) versus (23%) from the left ovary at P<0.05 (Najjar *et al.,* 2018). The present study agreed with the previous conclusions of Davies Morel and O'Sullivan (2001) and Rizagholizadeh *et al.* (2015) who stated that the two ovaries of the mare function alternately, and that there is no difference between their ovulation rates.

Conception rate to AI with frozen semen in this study significantly affected by number of service per conception (P<0.05) with a rate of 3/21(14.29%), 6/17(35.29%) and 6/9(66.67%) for one, two, and three services/conception respectively. Increasing the number of cycles that a mare is bred will increase the chances of particular mare becoming pregnant at the end of the season (Samper *et al.*, 1991). Sieme *et al.* (2003) indicated a significantly increasing foaling rates with number of insemination and concluded that fertility is improved by multiple inseminations. The number of service (cycle)/conception required in the present study showed an overall mean ($\pm$SEM) of 2.2$\pm$0.2, in which higher number of services/conception required for local breeds than cross breed mares with statistically significant difference (P<0.05). The overall average in the present study was higher than reported value of 1.43 (Gibbs and Davison, 1992), 1.24-1.5 (Cilek, 2009), 1.46$\pm$0.3 (Ali *et al.*, 2014) as average number of cycles or services needed for a mare to get pregnant. The differences of the results were described partly by Gibbs and Davison (1992) who demonstrated the effect of age, as oldest age group of mares requires more services per conception and the milking mares required significantly more cycles per conception in the breeding barn than did the mares that were in open status. Differences in body condition of studied animals was one of important factors, as poor body condition animals require more NSPC than good and more body condition scores (Freeman, 2014). Other scholars stated that, fertility of the stallion, fertility of the mares, value of the horses involved, and intensive veterinary management of mares as to brought significance differences (Meliani *et al.,* 2011).

Concerning the effect of preovulatory follicle size on pregnancy rate, a higher rate of 55.56% for follicle diameter of 38-45mm as compared to larger diameter (>45mm) with rate of 26.32% (Figure 4). This result agrees with the findings of Ginther *et al.* (2008b), Arbel *et al.* (2015) and Najjar *et al.* (2018). Preovulatory follicles with a diameter between 35 and 44mm gave a higher rate of pregnant mares compared to the ones with a larger diameter (>44mm) with 87% versus 23% respectively ($p < 0.05$) (Najjar *et al.,* 2018). In opposite to the above findings, follicle size is related to reproductive success as small pre-ovulatory follicle size may account, for the poorer reproductive performance (Shirazi *et al.,* 2002; Davies Morel *et al.*, 2010). The importance of larger diameter of ovulatory follicle expected for higher fertility as compared with less diameter of follicle is due to possibility of large CL development with subsequent increase in circulating progesterone concentration (Lucy, 2007). Mata *et al.* (2013) explain that, even size of the mature follicle at ovulation shows some variability, size of the follicle does not affect the pregnancy rate as long as it is a mature follicle and support the present findings.

9. CONCLUSION AND RECOMMENDATIONS

A closely similar preovulatory follicles and cross sectional uterine diameter were measured between the two breeds of mares; but cross breed had significantly higher number of follicles than local breed mares. A positive correlation of cross sectional uterine diameter, endometrial fold score and teasing scores with developing dominant follicles can have used to estimate the time of ovulation from these findings and hence can fix the best time for AI. A high conception rate to frozen semen was obtained in the present study with different number of services per conception between breeds. There was required higher number of services per conception for local breed than cross breed mares and more number of services per conceptions were again required for lower body condition score mares than higher condition scores to become pregnant. Therefore, the following points were forwarded as recommendations:

- ❖ Correlation of behavioral estrus with developing size of dominant follicle should be considered for determining of breeding time effectively in combination with ultrasonography of reproductive organs
- ❖ It is preferable to use postovulatory insemination in order to minimize the number of services per cycle due to delayed ovulation after preovulatory follicles reach maximum size.
- ❖ When valuable animals with expensive semen used, flushing the uterus is important, few hours after AI to reduce uterine irritation, reduce early embryonic mortality and improves conception rate
- ❖ Estrus and ovulation induction should be practiced in case of delayed ovulation; whereas evaluation of the effect of induction on fertility rate were highly recommended points as a research gap.

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11. ANNEXES

Annex I: Criteria for classification of teasing score

Score 0: Resists stallion, pins ears, vocalizes, kicks at stallion

Score 1: Indifferent toward stallion, tolerates presence of stallion

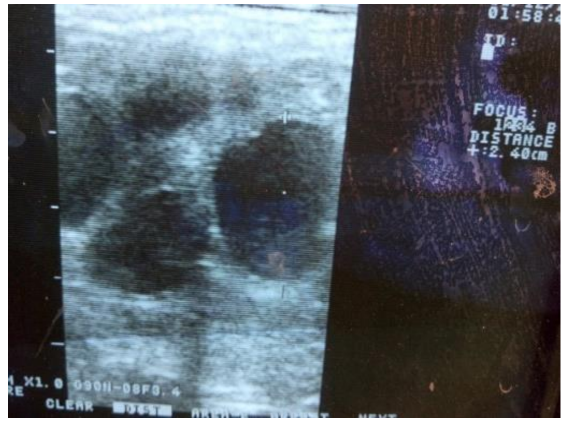
Score 2: Interested in stallion as evidenced by advancing toward stallion, lifting tail

Score 3: Stands close to stallion, sometimes in a squatting position, some urination and eversion of clitoris (winking)

Score 4: Squatting, frequent urination, eversion of clitoris, leaning toward or onto stallion

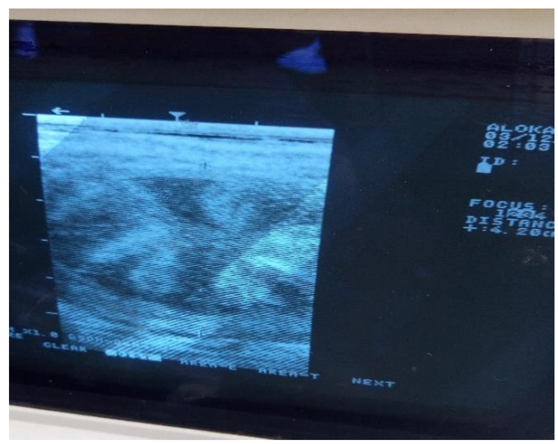
Annex II: Photo showing serial scanning of the mare's uterus, and ovarian structures by ultrasound

a) Ultrasonography of reproductive organs



b) Group of developing follicle

c) Preovulatory follicle



d) Developed endometrial fold

e) Embryonic vesicle on 15 days' post AI

Annex III: Photo showing teasing of mare

Annex IV: Artificial insemination of mare

a) Microscopic examination of sperm motility b) Artificial Insemination of mare

Annex V: Ethical clearance certificate

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ADDIS ABABA UNIVERSITY
College of Veterinary Medicine
and Agriculture
Bishoftu/Debre Zeit

Animal Research Ethical Review Committee

Ethical clearance certificate

Certificate Ref. No: VM/ERC/15/05/10/2018

Name of Applicant: Ararsa Duguma (DVM, MSc fellow)

Address: College of Veterinary Medicine and Agriculture, Addis Ababa University

Title of the project: Ovarian follicular dynamics and effect of breed on conception rate to artificial insemination with frozen semen in mares

Date of application:	**09/11/2017**
Nature of the project:	**non- invasive**
Target animal species:	**horses**
Number of animals involved:	**30**
Study area:	**Addis Ababa, Ethiopia**

Minutes No. and date of review: **VM/ERC/05/10/018, 03/01/2018**

The above indicated research project is acceptable from ethical perspective, relevance, originality and technical competence points of view. Hence the project is allowed to be executed provided that:

1. All procedures and conditions stipulated in the proposal are respected and any deviation or changes be reported to the committee
2. The project activities be open for occasional supervision by the committee whenever this is deemed necessary

Dr Getachew Terefe
Chairman

Signature

The Federal Democratic Republic of Ethiopia
Addis Ababa University College of Veterinary Medicine and Agriculture

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Please quote Our Ref. No. When replying

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