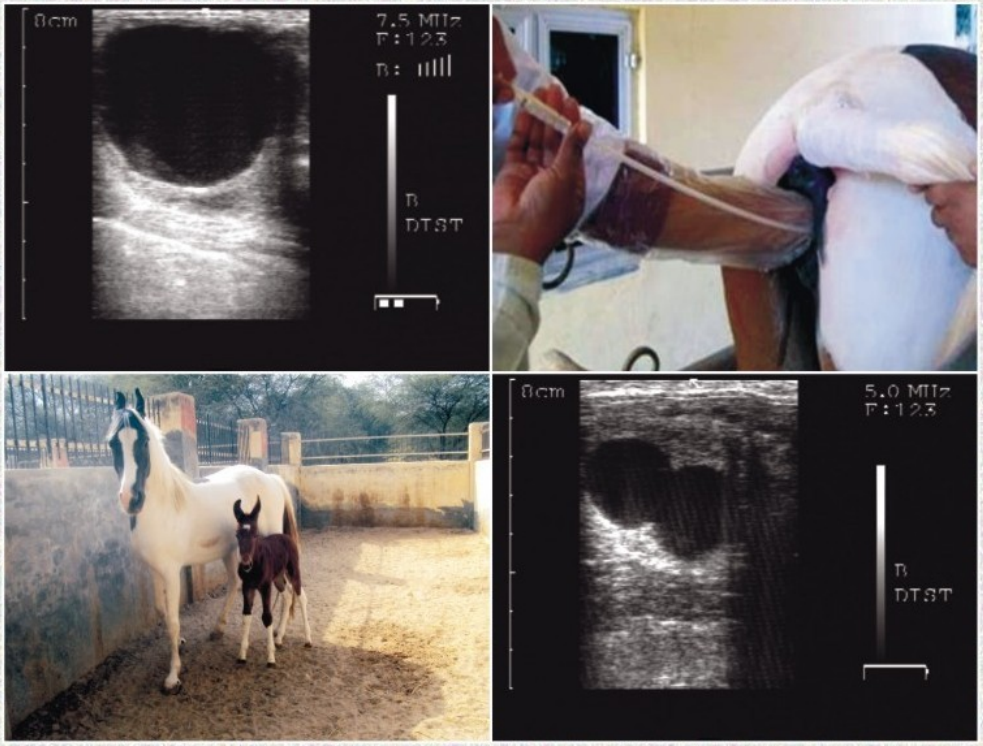
ICAR-NRCE Technical Bulletin

ARTIFICIAL INSEMINATION AND PREGNANCY DIAGNOSIS IN EQUINES



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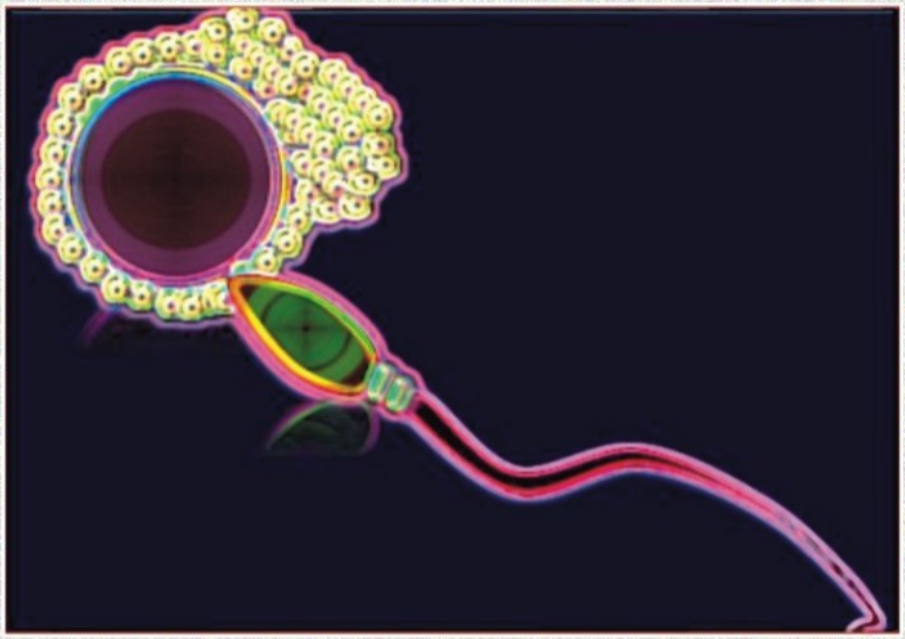

FOREWORD

India has a unique collection of geographically distinct, draught hardy indigenous breeds of horses along with local donkey population which are well adapted under varied agro-climatic conditions. Ponies, mules and donkeys are still a source of livelihood for socioeconomically poor and landless owners at many places in India. Since the equine population is decreasing rapidly due to their decreased utility and indiscriminate breeding practices, there is an urgent need to have a clearly defined "Equine Breeding Policy" to ensure sustainable improvement of indigenous equines through selective and scientific breeding and conservation. Indiscriminate breeding has already led to erosion of genetic resources in almost all recognized breeds of horses, viz., Marwari, Kathiawari, Manipuri, Spiti, Zanskari and Bhutia as well as donkeys. Thus, the conservation and preservation of indigenous breeds of horses and donkeys should be done in their home tracts as they are adapted to particular geo-agro climatic regions. Assisted reproductive technologies like semen cryopreservation and artificial insemination (AI) in equines could be an answer to address such issues and help in conservation of the equine germplasm. AI technique has been widely used in the domesticated animals worldwide for rapid genetic improvement and propagation of superior germplasm. AI technique facilitates an easy transport of semen and its storage for years by cryopreservation, and controls the transmission of venereal diseases to susceptible mares by coitus. The application of AI results in several fold increase in the utilisation of males with superior genetic merit and accelerates the introduction of new genetic material through the semen. The technique can be applied to enhance distribution of elite equine germplasm to farmers, who otherwise cannot afford to breed their animals with superior germplasm.

ICAR-NRCE has standardized the technique of AI in mares and jennies. The Centre provides the artificial insemination services free of cost to farmers in the field. AI for the production of mules has gained momentum in the states of Rajasthan, Gujarat and Haryana, while AI for foal production is at its pace in Rajasthan. The NRCE also provides the services of pregnancy diagnosis by ultrasonography and rectal examination in all the states, where Centre's team goes for animal health camps, animal fairs and kisan melas. The NRCE also provides pregnancy diagnosis services employing the Pregmare Kit as and when serum samples are received from field.

This technical bulletin, brought out through the experiences learnt over the years will be useful for academicians and researchers engaged in equine production using assisted reproductive techniques. The bulletin can be used as a teaching and training material to learn the process and techniques of artificial insemination and pregnancy diagnosis in horses and donkeys.

It is a matter of immense pleasure for all of us to present the technical bulletin on '*Artificial Insemination and Pregnancy Diagnosis in Equines*' for equine researchers, teachers and practitioners. The authors would be glad to receive critical feedback on the technical bulletin enabling us to further improve its content and quality.

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CONTENTS

1. INTRODUCTION

Leeuwenhoek (1678) and his assistant, Hamm, were the first persons to observe and identify the sperm, which they called "animalcules." Artificial Insemination (AI) by using these animalcules is the most fundamental and wide spread artificial breeding technique that circumvents physical or behavioral impediments to natural mating and provides the means for genetic exchange between populations without transfer of live animals. AI eliminates the epigenetic effects on the female gamete that are inherent in more invasive assisted reproductive technologies. It is a powerful tool for increasing the number of offspring produced by a single male, and for the transfer of genes between populations. In its simplest form, insemination of a naturally cycling, spontaneously ovulating female without disturbing or modifying the normal folliculogenesis, ovulation, luteinization, or the hormonal milieu surrounding these processes. For a successful conception post insemination, sperm must negotiate and interact with at least a portion of the female reproductive tract, promoting capacitation and normal acrosome responses to egg vestments. By definition, AI comprises the collection of semen from a male, followed by the transfer of that semen into a sexually receptive female prior to ovulation or immediately after ovulation in order to result in fertilization. AI is usually performed with fresh, chilled and transported and cryopreserved semen

The technique of AI is not new and being widely used in most of the domesticated animals worldwide for rapid genetic improvement. AI was the first assisted reproductive technique applied to control and improve reproduction as well as genetics. In India, the use of AI to breed mare is in preliminary stage and its potential is waiting to be explored. Indian Council of Agricultural Research -National Research Centre Equines (ICAR-NRCE) has taken lead to conserve the germplasm of indigenous equines through cryopreservation of semen of indigenous equine breeds and practicing AI since last two decades. ICAR-NRCE also provides free AI services to equine breeders especially in the states of Rajasthan and Haryana. AI with frozen semen is being practiced at ICAR-NRCE in different breeds of farm mares and Jennies which was extended further to mares visiting to the centre as well as in mares located at field level. The conception rate of 72.73% and 66.67% were reported in Marwari mares (Arangasamy *et al*., 2008) and exotic donkeys (Arangasamy *et al*., 2009) respectively, using fixed time insemination scheme with frozen semen. Encouraging results obtained from farm and the field with positive attitude of the farmers towards AI with frozen semen for the future use. Birth of foals with frozen semen insemination in field has also been reported from Rajasthan, Haryana, Punjab and Uttar Pradesh states in India. Frozen semen of Poitou

donkeys has been successfully used in field AI to produce mules with a pregnancy rate of 62%.

The option of having semen collected and frozen from the stallions for future use opens up a whole new dimension to the services which they can offer. Interest and necessity have rapidly grown to transform equine semen cryopreservation throughout the world into a successful industry. The use of flowcytometry and computer assisted semen analysis (CASA) have provided the researcher and clinician with powerful tools to evaluate several sperm attributes. These procedures have been utilized to evaluate sperm viability, acrosome status, mitochondrial status, DNA integrity and stages of capacitation. Several sperm attributes can be evaluated on thousands of spermatozoa in a matter of seconds with more accuracy. Continuous attempts to expand and increase the efficiency of the AI have concentrated on a range of aspects including low sperm insemination, and insemination technique and time. Success of AI requires that horse breeder understand the reproductive cycle in mares, handling/quality of semen, timely insemination, technician's skill, reproductive health of mare and fertility of stallion.

2. HISTORY OF AI

The use of AI has long been acknowledged as being acceptable in the cattle and sheep breeding industries, but it is only recently that horse breeders have begun to realize its potential. The history of AI itself is an interesting fact as according to some writers the earliest recorded semen collection and insemination took place way back in 1322 when an Arab chief used artificial methods for the successful insemination of a prize mare. Purportedly he used semen stealthily collected from the sheath of a stallion belonging to an enemy chieftain. There is no evidence, however, to indicate that the ancient tribesmen practiced artificial insemination in any appreciable degree. The first successful insemination was performed by the Italian physiologist and priest Abbe Lazzaro Spallanzani (1784) in a dog which whelped three pups 62 days later (Foote, 2002). The establishment of AI as a practical procedure was initiated in Russia in 1899 by Ivanov who studied AI in domestic farm animals, dogs, foxes, rabbits and poultry. He also developed semen extenders. Milanov, another Russian scientist and successor of Ivanov, started large scale breeding programs for cattle and sheep, and designed and made artificial vaginas. Horse breeding programs and research was initiated at the same time in Japan even though translations of the original research only became available to the western world after 1958. Frozen-thawed semen has first been used to inseminate mares in 1957 (Barker and Gandier, 1957) even though it only gained increasing popularity over the last 15-20 years. Pregnancy rate per cycle for frozen

semen varies between 30-50% on average (Vidament et al., 1997; Leipold et al., 1998; Sieme et al., 2003; Vidament, 2005; Metcalf, 2007).

3. POTENTIAL OF AI IN HORSES

In India, true to bred horse and pony breeds are endangered which necessitate immediate steps to be taken for their germplasm conservation and breed propagation. Semen freezing along with AI is valuable tool for germplasm conservation and breed propagation at a faster pace. The numbers of elite and pure bred indigenous horses are very few which cannot meet the demands for breeding the high number of mares. Use of AI increases the spectrum of such elite stallions to produce hundreds of foals over his reproductive life. There is limited sex drive of a particular stallion to breed mares in a day. Use of AI, on the other hand provides opportunity to breed more number of mares at same time on the same or different places. Cryopreserved semen from the valuable elite horses can be used for AI even after stallion is no more, ill, injured or participating in competitions. The use of equines particularly ponies and mules as draught power have special economic significance in difficult terrains in hilly, arid and semi-arid zones of India. AI along with semen freezing can be a valuable means of germplasm conservation and its propagation in current scenario of declining population of all indigenous pony breeds. Breeding mares to produce mules is source of livelihood for many and for them mule fetches a good market price. Indian donkeys measure less in height and face incompatibility to serve the mares in an attempt to have mule pregnancy. AI in mares with semen of donkeys is a good and an easy alternative solution to this problem.

Cost of breeding can be reduced significantly with the use of frozen semen AI which also allows breeding mare at desired time and the place which save cost incurred on transport. ICAR-NRCE has developed facilities for semen freezing and maintaining a limited stock of frozen semen doses for use in AI. This can be facilitated to a large scale production of quality frozen semen doses on behalf of Government or public participation under Government regulation. Export and import of frozen semen have a huge marketing potential. This is especially important for indigenous breeds of horses introduced to other countries and is isolated with a small genetic pool. Similarly, semen can be imported to prevent inbreeding and to expand the genetic pool. There is good future perspective for production of high quality frozen semen on commercial basis. Availability of quality frozen semen for AI at farmer's door will help to propagate purebred animals and to reduce the number of non-descript equine population. Marketing of sexed semen is another dimension of frozen semen with lot of market potential to be explored.

Stallions used to serve many mares are put at the risk of getting genital infections. The stallions with genital infection may spread the disease to served mares without showing the

clinical symptoms. Use of AI ensures routine semen evaluation and is safe as the antibiotics are added while processing the semen meant for AI. A control on sexually transmitted diseases can be achieved through strict disease monitoring of donors and by adopting a standard operating protocol for semen production. Import and export of horses involve long days of quarantine confinement and risk of disease invasion through potential harbours. However, risk of disease transmission across border is low on import or export of frozen semen for breeding purposes. In mares with exaggerated immunological response to spermatozoa that shows some degree of endometritis post coital can be benefited with smaller doses of spermatozoa used in AI than would normally be ejaculated at natural service. Acceptance of AI also provide basis for the development of other associated technologies such as cryopreservation, sperm sexing, low dose insemination, estrus synchronization, superovulation, embryo collection and transfer, culture and cloning. Thus, the impact of AI is much more profound than simply the other way to impregnate females. Limitations of AI include genetic pool reduction, expenditure on equipments for cryopreservation i.e. well furnished laboratory and storage facilities, require higher degree of knowledge and skill for proper collection, evaluation, storage of semen, thawing, handling and deposition of semen. Moreover, stallion sperm are fragile in nature and AI involves certain degree of human risk at time of semen collection.

4. ANATOMY AND PHYSIOLOGY OF MARE'S REPRODUCTIVE SYSTEM

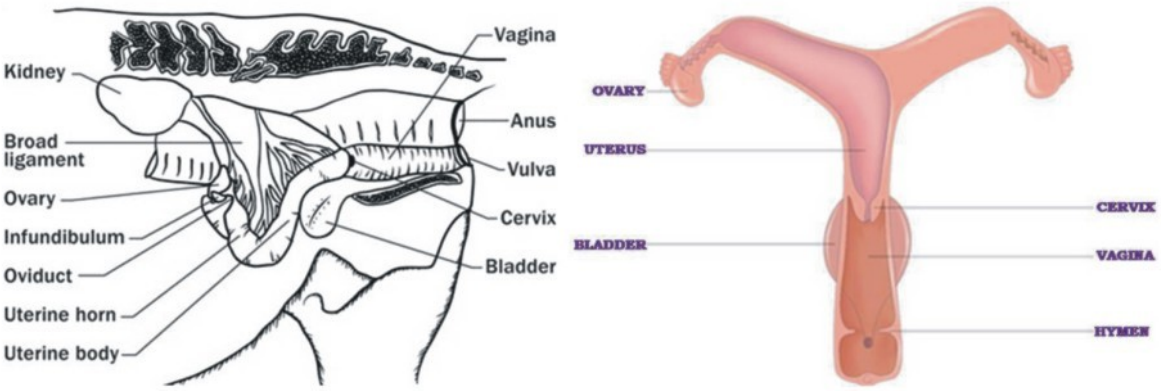


Fig. 1.Reproductive anatomy of the mare (Sagittal and vertical)

(Adopted and modified from https://veteriankey.com/the-mare)

A mare's reproductive system includes a vagina, cervix, uterus, oviducts and ovaries. The vagina is a flexible expandable collapsed lumen that takes up only one third of the

reproductive tract and is approximately 20-35cm in length. Another one third consists of the cervix and the left and right uterine horns. The cervix is 5-7cm long, but undergoes the most physiological changes during estrus and diestrus (out of estrus). Thick smooth muscle with elastic fibers creating longitudinal folds composes most of the cervix. During estrous the cervix expands allowing easy access for fingers to physically check the dilation of the cervix. This also leaves the uterus more vulnerable to bacteria and viruses. The uterus lies in the abdomen and consists of a shorter uterine body with right and left uterine horns. The main uterine body consists of longitudinal and inner circular smooth muscle (Causey, 2000). It is a collapsed lumen that can easily be inflated by air or fluids. The uterine body will contract and move sperm and semen towards a developed follicle in a uterine horn. The uterus contains mucosal folds which release large amount of mucous with hormonal changes.

4.1 Estrus and estrous cycle in mares

The mare is seasonally polyestrus and has a reproductive season and a non-reproductive season, both of which are controlled by light. The non-reproductive season, known as anoestrus, occurs in the winter season when there is insufficient day light. The reproductive season begins in the spring when light levels increase and continues through the summer. Mares therefore cycle naturally from March/April through to September/October. The peak of the breeding season is in the months of April, May, June and July. Two other periods are known as the spring and autumn are the transitional stages. But in tropical countries like India, the breeding season often start early i.e from the months of March and lasts till the months of October. One occurs just before the mare becomes reproductively active in the spring and the other occurs just before anoestrus in the winter. During these periods mares are generally erratic in their cycles and sexual behaviour. The spring transition period coincides with increased daylight hours, increased grass growth and ambient temperatures. As the season progresses estrus cycle in mare becomes regular. Puberty in the filly occurs, on average, at one-and-a-half years of age. Spring-born fillies show heat as yearlings and those born later in the year generally do not cycle until they are two years old.

Fig. 2. The breeding and non-breeding seasons for mares.

There are two different stages to the estrous cycle. These are generally distinguished by the mare's behavioral responses to the stallion. Estrus (heat) lasts an average of 5 to 9 days. Interestingly, the mare has the longest estrus period in comparison with any other domestic animal. Estrus is characterized by receptivity to the stallion. A mare showing classical estrous behavior will adopt an urination stance - squatting with legs spread and tail raised. She will lean towards the stallion, urinate in small volume frequently, and expose her clitoris by averting her vulva (winking) (Fig. A-C). Most mares cease estrus behavior within 24 to 48 hours following ovulation. This marks the beginning of the other stage of the cycle, known as diestrus, which lasts an average of 14-16 days. During diestrus, the mare rejects the stallion with behavior typically seen in the form of tail switching, squealing, striking, biting and/or kicking. These behaviorally defined divisions in the estrous cycle roughly parallel the events which are occurring in the ovary is termed the follicular phase and the luteal phase. However, these latter two phases are defined by the endocrine (hormonal) events punctuating the estrous cycle.

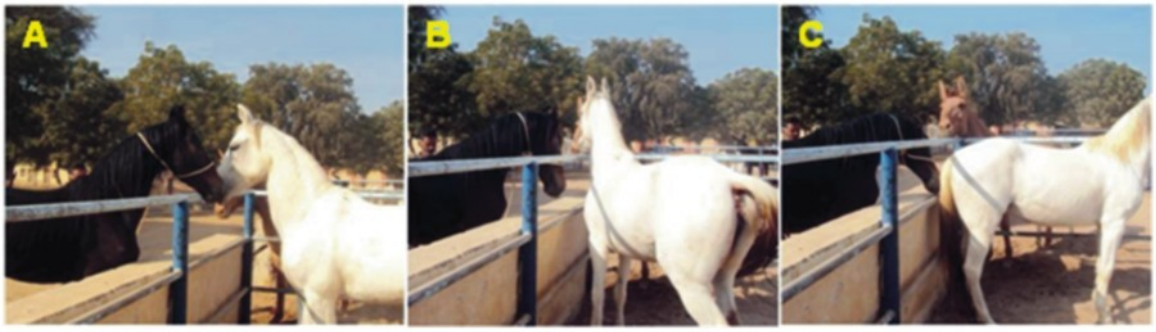


Fig. 3. Classical signs of a mare in Estrus.

4.2. Hormonal control of estrus

A mare is in diestrus when sexually unreceptive as opposite to “in estrus” or “in heat” when sexually receptive. Similar to stallions, photoperiods trigger the central nervous system and seasonal day length controls estrus: the pineal gland affects melatonin levels which affect the hypothalamus (Davies Morel, 1999). An increase in GnRH is a seasonal cue or “spring transition”. When mares in diestrus are approached by sexually excited males, they display aggression by pinning down their ears, striking, kicking, and clamping down their tail signaling “non-receptivity”. Recognizing physical signs is crucial in monitoring for insemination. A mare in estrus will have an excited reaction to excited stallions viz., licking, squatting, urinating, averting their clitoris (“clitoral wink”), and standing still; their vulva and labia become increasingly red, moist and smooth (Samper, 2000). Mares also round their back (called “kyphosis”) while most animals arch their back (termed “lordosis”) to signal their sexual receptiveness.

The hypothalamus gland begins the mare's reproductive season by producing gonadotropin-releasing hormone (GnRH), which stimulates the pituitary gland to secrete follicle-stimulating hormone (FSH) and begin estrus cycle for the 21-23 days. The season's first ovulation usually occurs 45-75 days after the initial GnRH surge or peak. During the cycle, FSH stimulates development of follicles in the ovaries until one or more follicles reach 30-45 millimeters in diameter. Estrogen produced by the follicles stimulates estrual behavior, shuts down FSH secretion, and stimulates the pituitary gland to release luteinizing hormone (LH). LH facilitates maturation of the growing, oocyte or egg bearing follicle, which ends in ovulation. Immediately after ovulation, the corpus hemorrhagicum forms in empty follicular cavity, which in turn becomes a solid body of luteal cells called the corpus luteum (CL) that produces progesterone. Progesterone is the key hormone in maintaining a pregnancy if the oocyte is fertilized, and it inhibits the secretion of FSH and LH from the pituitary gland. At this point, the mare goes into out of heat/diestrus. If the oocyte/egg is not fertilized, the uterus will remain under the influence of progesterone for 12 to 14 days, then changes will occur and the entire process will start over again. If the mare becomes pregnant, the presence of the conceptus will extend the life of the CL (and its production of progesterone) for 35-90 days, until the conceptus can produce progesterone on its own.

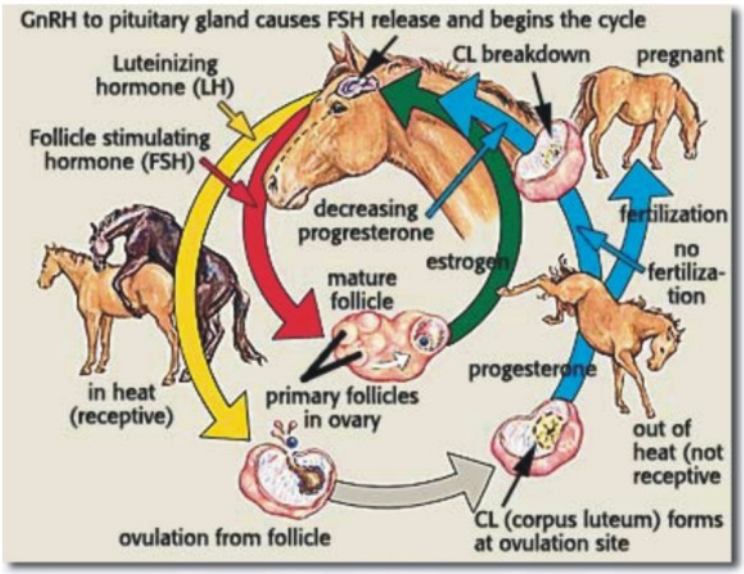


Fig.4. Hormonal control and sexual behaviour of mares in estrus.
(Adopted and modified from Robin Peterson illustration from Horse.Com)

4.3. Sexual Behaviour of mare and estrus detection

Accurate detection of estrus is essential to the efficiency of any horse breeding program. An important factor in successful reproduction of horses is the ability to determine when the

mare is ready to be bred. In order to breed the mare at the optimal time to achieve conception, it is imperative to be able to detect estrus, particularly if a technician skilled in rectal palpation and ultrasound monitoring is not available. For many, this simply involves recognizing estrus (the heat period) in mares so that they can be bred or inseminated at the appropriate stage of the cycle. However, many mare owners desire to have their mares foal at a specific time of the year. Conception rates are highest when mares are bred 1 to 2 days prior to ovulation. Although time of ovulation is difficult to predict without the use of ultrasonography, it most frequently occurs 24 to 48 hours prior to the end of estrus. Estrus generally has a range of 3 to 8 days. It begins with the mare showing a slight interest in the stallion and then increases in intensity until ovulation or shortly thereafter. Estrus ends abruptly one day after ovulation.

Palpation and teasing are the two best and most commonly used management tools being used in the detection of heat. Rectal palpation and ultrasonography will help define the time of ovulation, and thus aid in mating management. Parameters as follicular size, follicular consistency, cervical size and consistency, and uterine tone can be monitored through rectal palpation. A mare with a large, very soft follicle that has an open cervix is a right candidate for breeding. On the other hand, a mare that has no or very small turgid follicles and a closed cervix would be a poor candidate for breeding. Most managers use a combination of teasing and palpation for estrus detection and breeding determination. Mares are teased and those showing signs of estrus are palpated to better define reproductive status. Mares can be individually teased or placed in teasing pens which are constructed to allow for group teasing. Increasingly, ultrasonography is being utilized for estrus detection and determination of ovulation. With real-time ultrasonography, veterinarians can determine follicular size, early ovulation, uterine changes characteristic of estrus, and abnormalities of the reproductive tract. Ultrasonography is a significant contributor to reduction of the number of breedings or inseminations required per estrus. Each mare responds and acts differently during estrus. Thus, day to day monitoring of teasing status and reproductive tract parameters is essential.

For successful insemination, a mares' sexual cycles must be monitored or controlled. Simple monitoring is done by physical examination or electronically by ultrasonographic imaging (Samper, 2000). Internally, mares will have an increase in clear, highly lubricating, low viscosity vaginal and cervical fluid. The pH in a diestrus mare is approximately 7, the pH of an estrus mare can be more alkaline or more acidic due to changes in vaginal mucous (Samper, 2001). The cervix will appear flat, and both the uterus and cervix are swollen and soft (Samper, 2000). The cervical orifice will dilate; at the beginning of estrus it will be one finger width wide, and by the end 3 fingers‟ width wide. The uterine horns will easily flatten

with touch during palpation, as opposed to diestrus when uterine horns remain hard and incompressible (Samper, 2000). Endometrial folds will increase in number and become slippery.

4.4 Signs of mare not in estrus

- Rejection of stallion's presence (ears pinned, kicking, biting or pawing at stallion) or complete lack of interest in stallion.
- Not aggressive toward stallion, but looks away or is not interested.

Signs of a mare in Estrus/heat

- Shows interest by facial expression and may approach stallion; slow to show interest. Mare may raise tail or exhibit some winking (eversion of the labia of the vulva). Mare may show these signs at a distance, or in close proximity to stallion.
- More interest in stallion as demonstrated through facial expression, tail raising, flexion of pelvis (posturing), winking and clear discharges with urination.
- Intense interest in stallion as demonstrated by turning hindquarters to stallion, leaning towards stallion and exhibiting frequent winking and urination.

4.5 Factors that can affect the expression of estrus in mares

Mares with Foals: Mares may be protective to the foal and not exhibit estrus. It may require teasing the mare outside the stall/stable away from the foal (the mare may show a sign of estrus after the stallion leaves). And mares in lactation due to hormonal inhibition they may not exhibit the estrus symptoms.

Maiden Mares: Mares that have never been teased before will require additional time to become familiar with the process. In some situations with maiden mares, you may need to lead the mare to the teasing area in order for the mare to learn the process.

Timid Mares: Some mares may not show estrus when being actively teased by the stallion but may do so as the stallion moves away. In a stall or pen teasing situation, these mares may show strong signs of estrus when the stallion is teasing the mares before or after them.

Weather or Other Environmental Conditions: Hot or cold temperatures, wind, or precipitation may reduce signs of estrus. Also, the presence of insects may distract a mare and keep her from expressing true estrous behavior.

Stage of Breeding Season: Early in the breeding season, some mares may not exhibit signs of estrus as readily as later in the breeding season.

5. SEMEN COLLECTION

Semen collection using AV is an ideal method in equines. A dummy or estrus female is used for this purpose. In case dummy is not available and using an estrus female, her tail is bandaged; perineal area is washed and cleaned after proper restraining. The operator holding AV and the animal handlers should be alert and take necessary precautions to protect themselves from mare as well as stallion. Use of helmets during semen collection is an added precaution while stallion is mounting and dismounting. The penis of the jack/stallion is washed with luke warm water before mounting. Just after dismounting penis is given flush of mild betadine/antiseptic solution. Ejaculation is completed in 15 to 20 seconds marked by pulsation at the base of penis and flagging of tail.

Fig.5. Preparation of Artificial vagina and Semen collection from a Stallion

The AV is prepared by assembling its parts and filling hot water within the space provided through nozzle with help of funnel. The optimum temperature of AV is maintained 42 to 45°C and lubricated well with liquid paraffin or vaseline before use. Higher temperature of AV may cause irritation to stallion penis and cause damage to sperms. The pressure inside AV is such that the stallion is able to penetrate penis with ease. A pre-warmed graduated collection bottle covered with thermo-jacket is attached to one end with AV to accumulate an ejaculate and to prevent sperm from thermal shock. Ejaculation occurs into the lumen of the AV lined with a disposable plastic liner and is collected into graduated collection bottle. There are three major types of AV's such as Colorado, French (INRA) and Missouri model that satisfy these criteria in slightly different ways. Because of large size, comparatively heavier and vigorous thrust by the stallion at ejaculation, the AV used has a handle to hold it firmly.

5.1 Processing, storage, and handling of stallion semen

Evaluating the semen has diagnostic value in determination of cause, severity and the degree of pathological conditions of testes as well as accessory organs in addition to check

semen suitability for freezing. Good quality semen has predictive value for fertility of male. Semen should be evaluated as soon as possible after collection.

Semen is frozen into straws either by traditional vapor freeze technique or controlled rate freezer machine. Initial processing in both the methods is same. The semen samples having progressive sperm motility more than 60% in fresh is processed further for cryopreservation. The rate at which extended semen is cooled is critical. If the cooling rate is too fast or too slow, sperm viability is decreased. The straws loaded in controlled rate freezer machine were cooled at the rates of 0.3°C per minute from 18°C to 5°C; 10°C per minute from +5°C to 15°C and 19°C per minute from 15°C to 100°C. After reaching 100°C, the straws are taken out and finally plunged into liquid nitrogen.

Fig.6. Traditional vapour freezing of semen straws

Vapor freezing technique is used due to unavailability of bio-freezer being a costly instrument. The results obtained are comparable and satisfactory to automatic controlled rate freezer. In this technique, straws with diluted semen are laid horizontally onto a freezing rack and lowered into a styrofoam box that contains at least one inch of liquid nitrogen level. The freezing rack is designed to support the straws 3 cm above the liquid nitrogen level. After being held in that position for 12 minutes, the straws were then plunged into liquid nitrogen and stored at -196°C. During vapor freezing, the cooling rate at the bottom of the straw is generally much faster than at the top and to avoid variation in temperature the position of straws are turned around.

5.2 Dilution rate

The aim of dilution is to store semen samples at appropriate concentration and a sufficient volume for immediate insemination without any further treatment. Adequate

extension is ensure maximum sperm survival whereas excessive dilution results in low concentration that require large volume to be inseminated for acceptable fertilization rates. Usually 400-500 million progressive sperm per insemination/4 ml diluted semen is required in order to have optimal fertilization. The sperm concentrations of 200 millions/ml after final dilution with modified secondary extender are recommended. The recent development in horse AI is low dose insemination (5 to 100 million progressive sperm) to minimize the amount of semen used.

5.3 Packaging and storage

Mostly, the diluted semen is packaged into 0.5 or 0.25 ml straws. Some laboratories practice a single dose in 2.5 or 5 ml macro tubes before freezing. The volume of semen and size of packaging is related to convenience and the freezing success. There are specific cooling rates to perform while semen freezing and once frozen, the straws can be stored in liquid nitrogen (-196^{o}C) for an indefinite period.

6. FORMS OF AI

AI in horses is usually performed with fresh, chilled or frozen semen. Fresh semen is meant for immediate use in the mare at same place and is not much popular. Chilled semen is meant for insemination into mare within 24 hours of its collection whereas the frozen semen can be stored in liquid nitrogen for an indefinite period and can be used whenever the mare ovulates.

The length of time that sperm cells remain viable within the reproductive tract of the mare varies tremendously between fresh, cooled and frozen equine semen and varies with each stallion. Fresh semen will generally remain viable for at least 48 hours, cooled semen for 24 to 48 hours, and frozen semen only 12 to 18 hours. Therefore, the goal of breeding management of the mare is to deposit semen into the reproductive tract at a time that will maximize the probability that viable sperm cells are present at ovulation.

6.1 Fresh semen

This undergoes minimal or no processing and always has the highest fertility. It's long-lasting once inseminated, the inseminated mare requires less frequent veterinary checks prior to conception, and it's usually the least expensive method. However, as it can't be transported and must be used almost immediately, mares and stallions need to be at the same location for insemination. The semen collected from the stallion will be extended in skimmed milk and will be inseminated into the mare uterus immediately (within 1-2 hours) through AI. Fresh semen from a fertile stallion would survive in the reproductive tract of the mare nearly for about 60 to 72 hrs . Ideally the mare should be inseminated 1-2 days before

she ovulates. Fresh semen is ideal for mares of all ages, and is the preferable option for less fertile mares, problem mares, and older mares that are above 15 years old.

6.2 Chilled semen

Mare owners order chilled semen and can have it transported inexpensively overnight by carrier or postal services using Styrofoam boxes or the more cost-intensive Equitainer. It usually retains good viability for 24-30 hours plus an additional 24-48 hours once inseminated. Ordering, shipping, and delivery must be well-managed to respect the semen viability time constraints, and the mare owner must order new semen if the mare does not ovulate within 24-48 hours after insemination. Collected from the stallion, extended with a special diluent, and then cooled gently to 4 degrees in a special 'Equitainer'. Sometimes this semen is also shipped in special Styrofoam boxes. If the stallion is fertile and the semen is handled carefully then the semen should last for 1-2 days and retain good fertility. Chilled semen is suitable for most mares, and if the stallion has a good record of fertility then it is also suitable for sub fertile and older mares.

6.3 Frozen semen

Liquid nitrogen tanks can preserve frozen semen for years, which allows semen to be ready and in hand whenever your mare ovulates. Special transporters, known in the U.S. as dry nitrogen shippers, can deliver frozen semen worldwide, and new one-way shipping containers provide a less expensive and more convenient way to transport frozen semen. Even so, frozen semen remains the most expensive and least successful AI method. Once collected from the stallion is processed and frozen for future AI use. In order to be of reasonable quality of semen , the stallion has to be fertile, and a known good 'freezer' (not all stallion semen freezes well) and it requires delicate and meticulous handling. After thawing the frozen semen usually survives only for about 6 to 12 hours. Therefore to optimise the fertility of the frozen semen, the mare's reproductive tract needs to be examined by ultrasound many times during oestrus to determine the best time for (AI). The pregnancy rates for frozen semen range from 0-70% depending on its quality and the fertility of the mare. We do not usually recommend using frozen semen in problem mares, or those over 15 years old. However individual cases can be discussed, and if the mare has a foal at foot she is likely to be more fertile than a barren or maiden mare.

7. AI PROCEDURE

Once considered for AI, the mare is first secured into travis and back racking is performed. This is followed by cleaning of perineal area thoroughly first with water and then

most commonly using an iodine preparation such as Betadine. Tail is wrapped with sterile gauge and deflected to one side where it should remain throughout the insemination process to avoid contamination of the scrubbed area.

7.1 Preparation of semen for insemination

Once the mare is ready for AI, the frozen straws is picked up from the shipping container/storage container, dipped in water at 37° C for 30 second for thawing and loaded in a 5 ml sterile syringe. Usually eight to ten straws of 0.5 ml are thawed and cut to make an insemination volume of 4-5 ml to have sufficient number of progressive motile sperm (400-500 millions) per insemination (Fig. A -C).

7.2 Thawing procedure

To use frozen semen for AI, straws are taken out from storage container, dipped in water bath kept maintained at 37°C for 30 second to 1 minute or thawing kit adjusted to 37°C In equines, large volume is needed for insemination so 8-10 frozen straws (0.5 ml) are taken out for thawing to make 4-5ml of volume. Straws are wiped with tissue paper to remove water and cut to load into syringe for insemination (Fig. A-E). A small drop from thawed semen is taken for evaluation of post thaw motility before use in AI. Semen samples having post thaw otility ≥35% is considered suitable for use in AI.

Fig.7. Removal and thawing process of semen straws

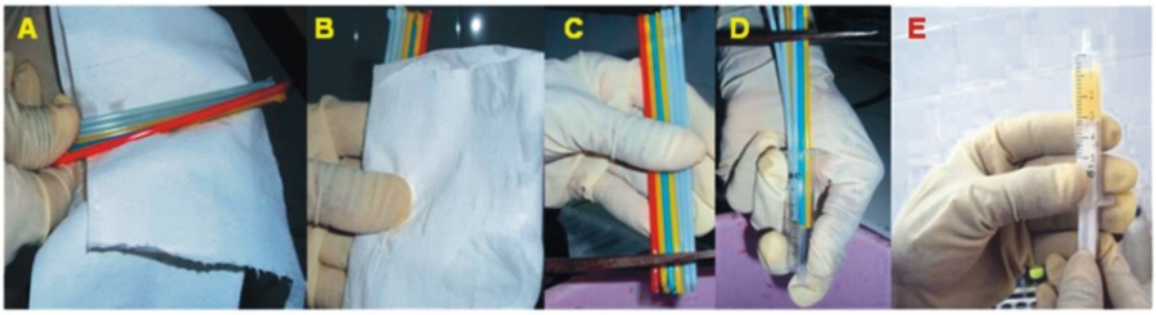


Fig.8. Cleaning and loading of semen from straws in to the inseminating syringe

7.3 Time of insemination

As ovulation approaches, the follicle feels very soft in per-rectal examination. The irregular shape of ovulating follicle with an ovulation point and size reaches between 45 to 55mm can be seen using ultrasound scanner. This helps in predicting the ovulation time and to decide the time of AI. Optimum time to inseminate the mare with fresh, chilled and frozen semen is 24 to 48 hrs before ovulation, 12 to 18 hrs before ovulation and 6 hours before to 6 hours after ovulation, respectively.

7.4 Frequency of insemination

Two or more AI with frozen semen per cycle usually results in higher pregnancy rates as compared to mares inseminated only once. Insemination need to be repeated at every other day when chilled semen is used or daily with frozen semen unless palpation or scanning of mare's ovaries is undertaken.

7.5 Dose and site of insemination

Usually eight to ten straws of 0.5 ml are thawed and cut to make an insemination volume of 4-5 ml to have sufficient number of progressive motile sperm (400-500 millions) per insemination. A total of 300 million progressively motile sperm per dose is also recommended for frozen-thawed semen. Post-thaw sperm motility in frozen semen samples should have at least 30% or more progressively motile sperm to be considered acceptable for AI in mares. Standard practice is to place semen in the uterine body. However, deep intrauterine AI with only 5 to 100 million progressively motile sperm may achieve an acceptable pregnancy rate.

Insemination techniques using low sperm numbers : As stated earlier, the current thinking is that the number of sperm reaching the oviduct may be only between 100 and 1,000. Once this discovery was made, it seemed a logical conclusion for researchers to investigate how few sperm may be necessary to achieve fertilisation when placed directly at the tip of the uterine horn during insemination. This interest stemmed from the increase in frozen semen usage. Semen from some stallions is notoriously difficult to freeze or in very short supply, and it would be of tremendous benefit if mares could become pregnant via inseminating much lower doses of sperm. The technique is termed low-dose insemination, and since the sperm are inseminated much further into the uterus than with conventional AI, the other term used to describe the technique is deep uterine insemination (DUI).

There are two methods for inseminating the sperm deep into the uterus. The first is to use a special catheter, which is guided up the uterine horn by placing one hand in the rectum of the mare and slowly advancing the catheter to the tip of the horn. The second method is to

place an endoscope in the uterus and visualise the tip of the uterine horn. The semen can then be inseminated via a special catheter inserted down the channel of the endoscope. These two techniques will now be examined in more detail.

7.6 Deep uterine insemination using a rectally-guided catheter

The mare should be prepared for DUI in a clean, well-lit environment; stocks for restraint are essential. Light sedation may be useful in certain cases. The tail should be bandaged and tied out of the perineal region. Immediately prior to insemination, rectal examination should be performed to empty the mare's rectum of faeces and confirm either the presence of a large follicle about to ovulate or the site of a fresh ovulation.

The inseminator must decide the side of insemination (left or right) based on this rectal examination. The vulva and perineal area should be thoroughly cleansed with very dilute antiseptic solution or mild soap. This is then thoroughly rinsed off with fresh warm water and the perineal area dried with clean, soft, disposable (paper) towels. The inseminator should use a sterile obstetric glove (such as a glove turned inside out). In certain circumstances, a sterile surgeon's glove should be placed over the clean rectal glove. It may be necessary to place a small amount of sterile, nonspermicidal lubricant (such liquid paraffin) on the top of the hand around the knuckles.

A special catheter is used, which is long enough (75cm) to reach the tip of the uterine horn while still having one end protrude from the vulva. The catheters have a special rounded tip at the end so that they can be advanced up the uterine horn ipsilateral to the ovary with the large follicle or fresh ovulation, without catching on the folds that line the uterus. The catheter should be held with the tip behind the fingertip and the hand brought into the vulva. The external opening of the cervix should be located with the index finger and a finger inserted into the cervical canal.

The catheter is inserted alongside the finger and gently pushed forward. It is very important that the catheter reaches into the uterine body and does not remain obstructed in the cervix. This passage through the cervix is not always easy. The hand used to introduce the catheter is then withdrawn from the vagina and placed into the rectum of the mare. The catheter can then be felt within the uterus and guided deeper into the uterus than can normally be achieved.

In fact, the catheter should be gently pushed until the tip is at the very tip of the uterine horn when it will be adjacent to the oviductal papilla. This is where the sperm should be deposited. The first straw of semen should be inserted into the catheter with the cotton plug towards the outside of the catheter. A steel plunger is used to push the straw to the tip of the catheter, where the open end lodges in the nipple-like protrusion at the end of the catheter. If

more than one straw of semen is used for the insemination, the system described above provides an easy and effective way of delivering the semen by removing empty straws from the catheter without having to replace the catheter. Using this technique, satisfactory pregnancy rates have been achieved with sperm numbers of 50 million to 100 million.

7.7 Deep uterine insemination using an endoscopic technique

It is possible to place an endoscope into the uterus of a mare through the vagina and cervix in much the same way as an insemination catheter is passed. The rectum is evacuated and the side of insemination determined, in the same way as for the rectally guided catheter technique.

The perineal area is washed and cleaned and the mare lightly sedated. A 1.6m to 2m flexible video-endoscope, with a diameter of at least 11mm, is used. The endoscope is inserted into the uterus of the mare via the cervix. Air is then passed through a channel in the endoscope into the uterus, which allows the inside of the mare's reproductive tract to be visualised.

The operator can then gently steer the tip of the endoscope all the way up the uterus until the entrance of the oviduct into the uterus is reached. This area is termed the oviductal papilla. A special narrow catheter can be passed down a central channel within the endoscope. The tip of the catheter is exposed beyond the end of the channel so it can be visualised. By carefully steering the endoscope's tip, the catheter can be placed either very close to or touching the oviductal papilla.

Semen is then blown out of the catheter directly on to the papilla's surface. Using this technique, acceptable pregnancy rates can be achieved using as few as five million sperm. This represents a 100-fold decrease in the usual number of sperm needed. By depositing the sperm so close to the site of fertilisation, the distance the sperm need to travel is reduced. In addition, exposure to the potentially hostile uterine environment is reduced. These are the two most likely reasons why sperm numbers can be so drastically reduced.

7.8 Insemination Procedure

The actual process of artificial insemination in a mare is not complicated, and can be learned very rapidly. Unlike artificial insemination in cattle, i.e, recto-vaginal method, in equine it is vaginal method of insemination. During AI perineal area is cleaned thoroughly most commonly using an iodine preparation such as "Betadine. Mare should be properly restrained. Tail is wrapped with sterile gauge and deflected to one side where it should remain throughout the whole insemination process to avoid contamination of the scrubbed area (Fig. 9. A-D).

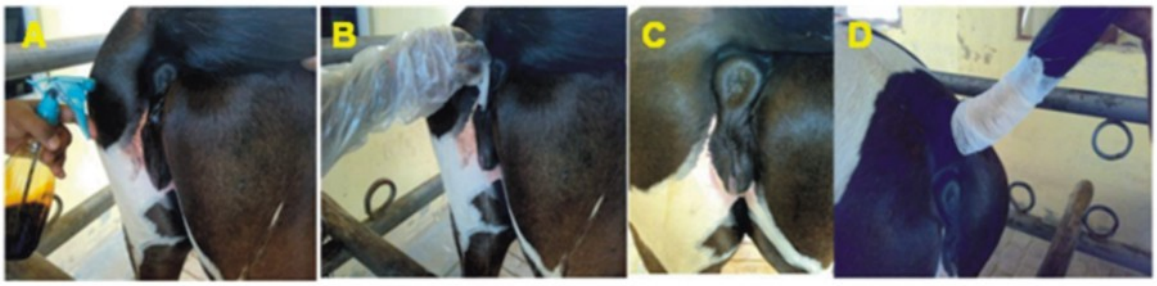


Fig.9. Cleaning of mare perineum before subjecting for AI

Once the mare is ready for AI, the semen is removed from the shipping container/LN_2 container and thawed at 37° C for 1 minute and loaded in a sterile syringe. The inseminator will introduce his arm into the mare's vagina after applying proper lubricants (non-spermicidal lubricating jelly), gloved thumb is placed over the end of the pipette prior to it's introduction into the vagina, palpate the cervix, which should be found on the ventral surface of the vagina (Fig. 10 A-D).

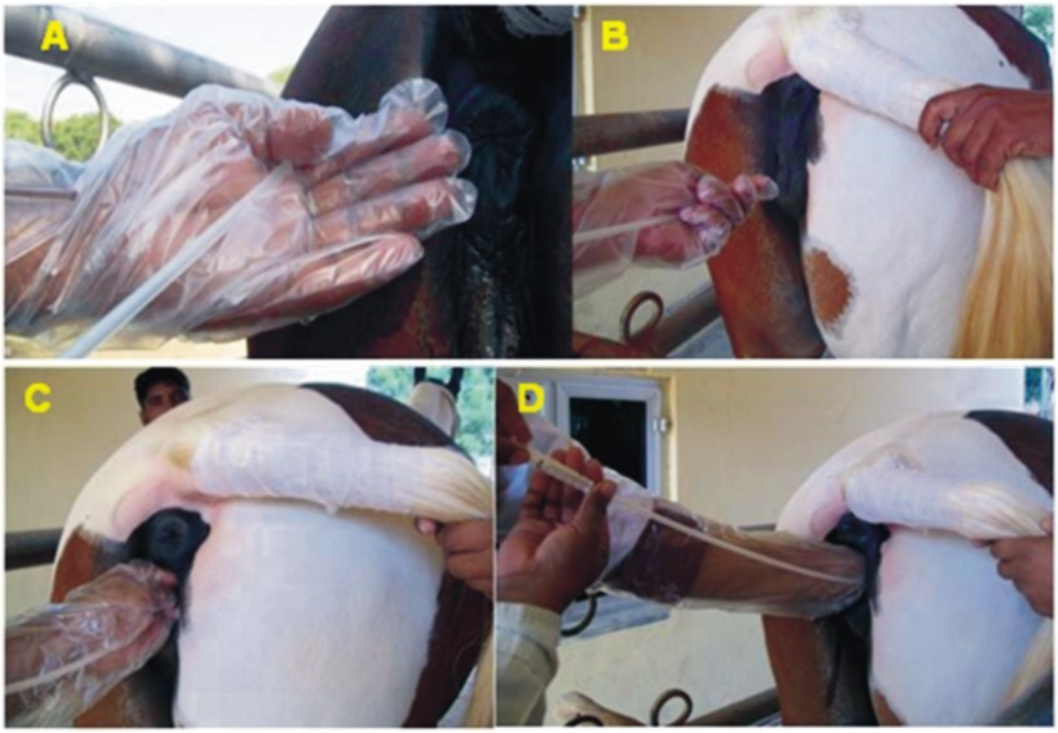


Fig.10. Steps for proceeding for AI

The closer the mare is to ovulation, the more relaxed the cervix becomes. In the center of the cervix will be found a small depression, which is the opening to the uterus after locating the cervical opening using index finger as shown in the following picture (Fig. 11).

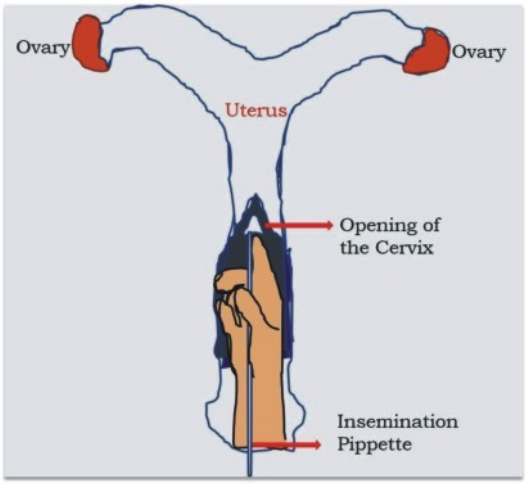


Fig.11. AI procedure in equines

It is important not to force the pipette at any point, as internal damage may occur if that is done. The gloved thumb is placed over the end of the pipette prior to it's introduction into the vagina. This protects it from picking up any contaminants which may subsequently be inseminated into the uterus along with the semen. With the pipette introduced into the uterus as far as possible without any resistance, the plunger of the syringe is slowly depressed, introducing the semen. Before removal of the pipette, the syringe may be unhooked and rehooked so that 2 cc of air may be introduced behind the semen in order to clear the pipette of the remaining semen. It is important that excess air is not introduced into the uterus, and a very small portion of the semen should remain at the very end of the pipette when it is removed from the uterus. The arm should then be slowly withdrawn from the vagina. It is acknowledged that the best pregnancy rates are achieved when the semen is inseminated no more than 12 hours before or 6 hours after ovulation. Consequently, it has been suggested that monitoring by rectal palpation should be performed as often as every 6 hours, or by ultrasound every 12 hours.

7.9 Factors influencing success of AI

Several factors affect the success of AI, including method of semen storage, the volume of semen and concentration of sperm in semen to be inseminated besides other considerations such as timing and frequency of insemination per estrous cycle. The technique and skill of the inseminator and fertility of the stallion or seminal quality also have an influence on the success of AI. It is acknowledged that the best pregnancy rates are achieved when the frozen semen is inseminated no more than 12 hours before or 6 hours after ovulation. Consequently, it has been suggested that monitoring by rectal palpation should be performed as often as every 6 hours, or by ultrasound every 12 hours.

Conclusion

There are advantages and disadvantages involved with all techniques as with the AI technique. The advantages of AI in equines far ever than the disadvantages, and that many of the later can be addressed by applying appropriate regulation, training of personnel for correct use of the AI technique and good management of associated activities.

Factors which have a great impact on any artificial programme are the quality of semen, the breeding status of the mare and the management of the mare during the estrus period. During semen preservation the quality of the semen is severely compromised. Beside these this the contamination with pathogens and saprophytes severely hamper the outcome of artificial insemination.

8. PREGNANCY DIAGNOSIS IN EQUINES

In equines, pregnancy diagnosis is of great economic importance for equine breeders and equine owners. It is known that equines are seasonal breeders and it is also well known that in equine breeding season is limited along with lengthy gestation period (Talluri et al., 2016). If a mare remains uncovered or remains non-pregnant during the breeding season due to false presumption of pregnancy or loss of pregnancy and is not covered again, then the loss of one breeding season along with the maintenance cost of the mare, is of great loss to the equine owners. Thus all the mares need to be examined properly for pregnancy at the earliest possible after breeding. Economic pressures of the equine breeding industry result in a demand for accurate early pregnancy diagnosis. Some horse owners consider that if a mare that fails to come back into heat (estrus) within 21-27 days after breeding is pregnant. That is not necessarily true. Up to 10% of bred mares that fail to return to heat are not pregnant. Reasons for a non-pregnant mare not coming back into heat include persistence of the corpus luteum (pseudopregnancy), lactational anestrus, seasonal effects on ovarian function, and some ovarian disorders. Besides early pregnancy diagnosis, pregnancy follow-up is also important in mares as early embryonic deaths accounts for 8 to 15% losses in equines (Roberts, 1980, Wood *et al.,* 1985). Thus, accurate pregnancy diagnosis is very important for management and husbandry reasons. In equines, rectal examination, ultrasonography and hormonal assay based techniques using urine, saliva and serum have been used widely for pregnancy diagnosis. Either of the method can be used depending upon the facility available with the equine owners. The most reliable and accurate test for the diagnosis of the pregnancyis ultrasonography. Pregnancy tests based on hormone levels in blood or urine are generally used only when palpation and ultrasonography per rectum is not possible or available.

As with traditional pregnancy testing in large animals, ultrasounds involve an "internal examination" via the rectum of the mare. The ultrasound probe is held in the hand of the Veterinarian and is directed over the uterus and ovaries of the mare, to produce a picture on the ultrasound screen. This is the only way to get a definitive result in early pregnancy (from 2 weeks to 4 months post mating), but later in the pregnancy, ultrasound examination through the flank of the mare may also be done. However, due to the large amount of intestines in horses, flank ultrasounds may not be as reliable as rectal ultrasound. There are also blood tests available, but they may also be unreliable, and definitely less reliable than rectal ultrasound.

8.1 Reasons for early pregnancy diagnosis

- It is very important to be able to tell if a mare is pregnant for management and husbandry reasons
- Pregnant mares should be managed differently from non-pregnant mares.
- Pregnant mares will require further examinations to monitor the development of the pregnancy.
- It is important to confirm the absence of twin pregnancies and monitor for early embryonic or foetal death.
- It is just as important to diagnose the non-pregnant mare as soon as possible. Some mares have prolonged luteal activity (previously termed 'persistent corpus luteum' (PCL)) and the maintenance of circulating progesterone concentrations in these non-pregnant mares means they do not return to estrus themselves.

8.2 Use of ultrasound for pregnancy diagnosis in the mare

Using ultrasound enables detection of pregnancy as early as 11 days post-ovulation. Most veterinarians, however, prefer to check mare between 12 and 15 days post-ovulation, when the embryonic vesicle is larger and easier to find. The experienced veterinarian will trace every nuke and corner of the uterine horns and body to find the early embryo (normally millimeter in diameter) and to rule out pregnancy and twin pregnancy. Ultrasound also allows monitoring for normal growth and health of the embryo. If early embryo loss is detected, one can arrange to rebreed the mare before the end of the breeding season. A peculiar character of pregnancy in the mare is that the intra uterine migration of the early embryo between both the horns and body, from days 7 to 16. This is a very important event in mare for *maternal recognition of pregnancy*; the embryo is asserting its presence by preventing prostaglandin release from the endometrium (uterine lining), which would induce the mare to come back into heat, and thus the pregnancy would be lost. This is why the embryo can be located anywhere in the uterus, when we perform early pregnancy diagnosis

(before day 16). At around day 17, the embryo becomes get lodged at the base of one of the uterine horns, where further development of the embryo will continue.

8.3 Stage of recommendation of use of Ultrasonography

Pregnancy can be detected as early as 11 days after service, but it is recommend that the earliest to be confident of the result is at 14-16 days after the last service. The actual date of "ovulation" (when the mare's ovary releases the egg into her uterus for fertilisation), which can also be detected by ultrasound, is the date which determines when the first pregnancy ultrasound can be done. Usually 14 days after "ovulation" the pregnancy can be readily detected by ultrasound, provided it is done in the right conditions. This coincides with about 14-16 days after last service if the mare was teased off after the last service.

Traditionally, most stud managers and mare owners would wait one cycle, or 21 days post service, to see if the mare came back into season, before having the first pregnancy test. However, one of the great advantages of ultrasound is early detection of twins. Twins are a headache in mares, as most twin pregnancies result in early foetal loss (slipped pregnancy), late term abortions, or birth of one or two undersized foals, and birthing difficulties for mares if they try to deliver twins. Twins are more likely in some breeds, with Thoroughbreds having by far the highest incidence of twins, but other breeds can also be affected. Twinning tends to be repeated in some mares, often with more fertile stallions also. Twins in mares are rarely identical, and therefore usually come from the mare having a double ovulation (releasing two eggs), rather than from the embryo splitting after fertilisation (identical twins) The reason why detecting twins as early as possible, ie at 14 days of pregnancy, is important, is because if the twins settle in the same horn(side) of the uterus (called kissing twins), there is only a short period of opportunity, from day 14-16, when one can be easily "pinched off" without risking lose both embryos. If the twins settle in separate horns of the uterus, one can be removed up to day 26 of pregnancy without risking loss of the other. However, until you ultrasound the mare, you don't know if she is pregnant at all, or whether she has twins, or where they are.

In the early pregnancy in the mare, there are three phase we recognise:

1. The mobility phase-this is up to day 16, and during this time the embryonic vesicle moves up and down the uterus.
2. Fixation phase, from day 16 to 35, when the vesicle attaches to the uterine wall, and during which time the embryo can be seen growing inside the vesicle.
3. Implantation phase, beyond 35 days, during which time the placenta has cells invade the wall of the mare's uterus to implant the pregnancy.

On busy horse studs, the routine approach is to do the first ultrasound before the end of the mobility phase, i.e. 14-16 days post ovulation. Depending on the health of the pregnancy

and the history of the mare, the next ultrasound would be 1-2 weeks later, in which time the developing embryo is clearly seen and the heart beat is a visible indicator of life. Some mares lose their pregnancy after 16 days, and if this is detected early, the mare can be treated and sent back to the stallion for another try. A third pregnancy ultrasound is usually done at or around 6 weeks of pregnancy, or any time after 35 days when implantation occurs. Any further pregnancy ultrasounds are usually only done if the mare has a history of problems, or if visible problems develop. In miniature ponies, sometimes only flanks ultrasounds can be done, and they should only be attempted after 4 months of pregnancy, as any earlier are often inconclusive. Ultrasound has greatly improved our ability to determine pregnancy early in mares, but to get the best results, and therefore the best value for money, we need to be doing it under the best conditions. Broad daylight on an ultrasound screen will greatly reduce our ability to detect subtle changes, and may cause twins to be missed in an early pregnancy.

8.4 Diagnosis of early pregnancy

***Day 10-11*:**

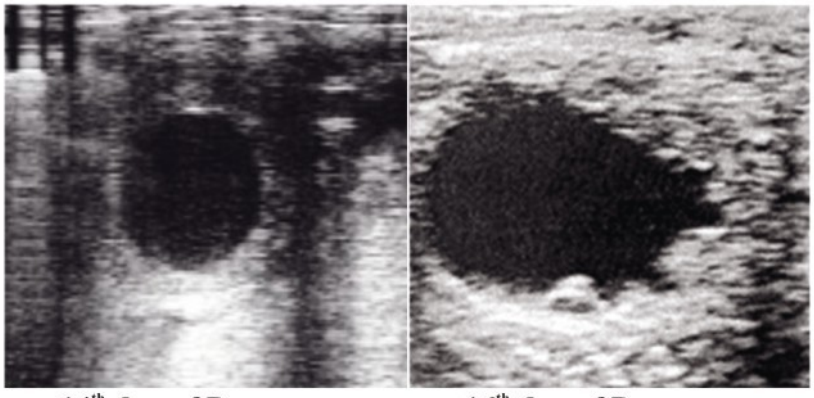
The equine embryonic vesicle can be reliably detected at day 11 when sufficient anechoic yolk sac fluid has developed. Mares are not usually scanned as early as Day 11 because it is possible to miss the conceptus if scanning conditions are not ideal and the ovulation date is not accurately known. If there was an ovulation one or two days after the first ovulation, any pregnancy arising from this later ovulation would be too small to be detected.

Day 14

The 14 day conceptus is 13 to 18 mm in size and lies centrally in the uterine body. Although pregnancy diagnosis is highly accurate even at this early stage, it is important to be aware of the possible confusion caused by uterine cysts and the presence of twin conceptuses. Ideally one would have performed an ultrasound examination before breeding the mare, but this is not always possible. If the first scan is performed at Day 14 or 15, then it is possible to return the next day in cases of confusion and see if the pregnancy has changed position or grown in size. This should allow differentiation from a cyst before the pregnancies have a chance to become unilaterally fixed.

Day 16

By day 16 of pregnancy the vesicle is normally fixed at the base of either the left or the right horn. The shape is still regular, but more ovoid than strictly spherical.



14th day of Pregnancy **16th day of Pregnancy**

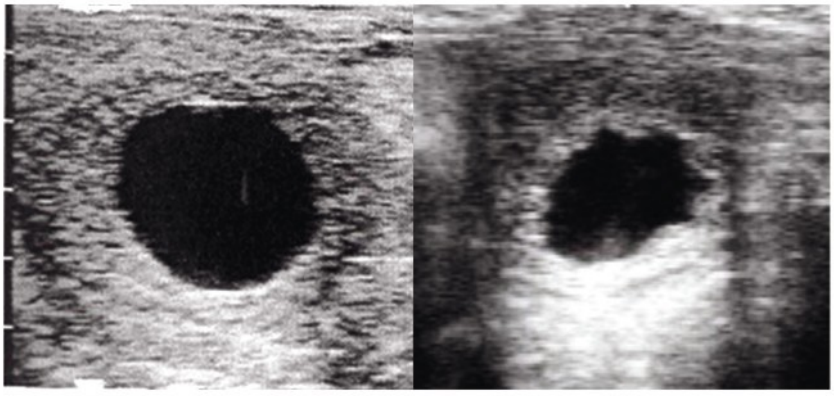
Day 22

At day 22 of pregnancy the conceptus is irregular in outline. This irregular shape is normal and is not an indication of imminent pregnancy loss. It is important to have an accurate history of the age of the pregnancy because small for age vesicles can indicate impending early embryonic death. All embryos should be detectable by day 24.

From this stage of pregnancy onward, morphological changes are also used to age the pregnancy. These include shape changes, the location of the embryo within the trophoblast and the relative sizes of the yolk sac and allantois.

Day 24

By day 24 of pregnancy, the heartbeat can normally be detected as a flickering movement in the middle of the echoic embryo around this stage of pregnancy. There is emergence of the allantoic sac as a small anechoic area from beneath the embryo. Over the next few days, the development of the allantois will lift the embryo dorsally and the yolk sac will gradually reduce in size. This change in ratio of the yolk sac to allantois is an important feature in ageing pregnancies. It is important to recognise the embryo and identify a heart-beat because the irregular shape of the vesicle is easily confused with an endometrial cyst.



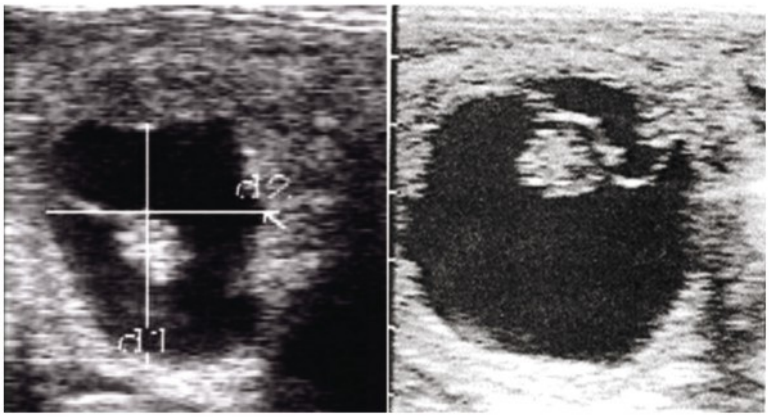
20th day of Pregnancy **22th day of Pregnancy**

Day 28-29

In the day 28-29 pregnancy the opposition of yolk sac and allantois results in an ultrasonically visible thin line normally orientated horizontally. The embryo is visible as an echoic mass on this line. There is enlargement of the allanioic sac such that the two sacs are approximately equal in size. The embryo is highly echogenic and is visible on the line separating the allantoic and yolk sacs and the heartbeat can be clearly seen.

Day 33

By day 33 the embryo is usually in the dorsal part of the vesicle often at a "2 o'clock" position. The volume of the allantois greatly exceeds that of the yolk sac. The aim of this examination is to confirm that a single conceptus is developing normally. If there is failure of normal development or if twins are detected, it is usually possible to terminate the pregnancy and re-breed the mare. However, if examination is delayed until after day 33, the endometrial cups may have developed and even if pregnancy is terminated, eCG production may continue for a variable time, sometimes preventing normal oestrous cycles for the rest of the breeding season.



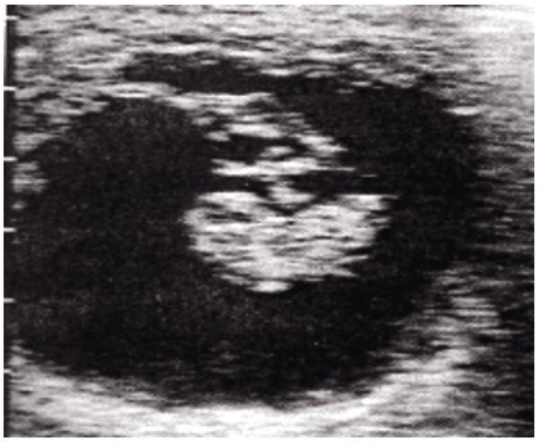
28th day of Pregnancy **33th day of Pregnancy**

Day 40

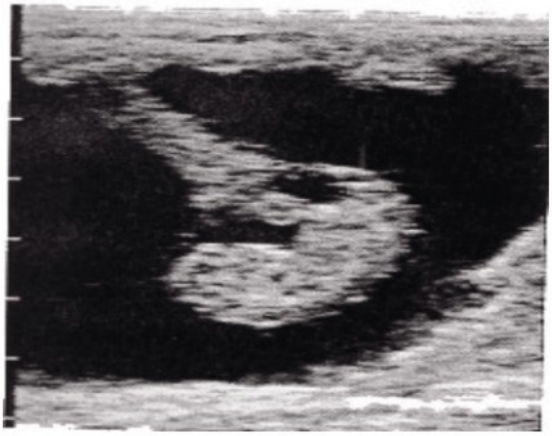
By day 40 of the pregnancy the allantois surrounds the yolk sac which has almost disappeared. The allantoic membranes are nearly opposed. The embryo is approximately 17 mm in length.

Day 45

By day 45, the foetus has descended approximately two-thirds of the way towards the ventral part of the allantois. The developing head of the foetus is recognisable.



28th day of Pregnancy



33th day of Pregnancy

Day 60

By day 60 of pregnancy the diameter of the conceptus exceeds the scanning width of the ultrasound probe. Considerable foetal motility is obvious.

8.5 Hormonal Tests for Pregnancy

Measurement of progesterone in the blood of mares is of limited value for pregnancy diagnosis since normal diestrous mares, pseudo-pregnant mares and some non-pregnant mares with ovarian abnormalities may all have elevated levels of progesterone. In contrast, a very low progesterone level (less than 1 ng/ml) indicates that the mare is very unlikely to be pregnant. Progesterone measurement is valuable in determininttg if a pregnant mare is producing sufficient progesterone to maintain her pregnancy. Normal progesterone levels vary with the stage of pregnancy so interpretation of progesterone results and management decisions must be made appropriately. Detection of elevated levels of equine chorionic gonadotropin (eCG) in the blood of mares may be used as a method of pregnancy detection. This hormone is produced from groups of specialized placental cells called endometrial cups and is only present in pregnant mares from approximately 35 to 120 days of gestation. Two major problems exist with using eCG levels for pregnancy diagnosis. First, a false negative diagnosis (i.e. no eCG detected in a mare that is truly pregnant) can be made if a blood sample is collected prior to day 35 or after day 120 of pregnancy. Second, a false positive pregnancy diagnosis (i.e. elevated eCG detected in a mare that is truly not pregnant) can be made if a blood sample is collected from a mare that lost her pregnancy after endometrial cup formation. Therefore, detection of elevated eCG in the blood of a mare will only confirm that endometrial cups are present and do indicate true pregnancy status or fetal health. Estrogens have also been used to determine the pregnancy status of mares. The estrogenic hormone Estrone sulfate may be used to diagnose pregnancy after approximately 60 days of gestation.

Small amounts of estrone sulfate are initially produced by the ovary of the pregnant mare in response to rising levels of eCG. Larger amounts of estrone sulfate are produced by the fetal-placental unit after day 90 of gestation. Measurement of estrone sulfate after the third month of gestation is useful to both diagnose pregnancy and monitor fetal viability as fetal death leads to a rapid decline in estrone sulfate levels. In summary, ultrasonography is the most useful tool for early pregnancy diagnosis, identification of twins, and detection of uterine or ovarian problems. However, if endocrine tests are required for pregnancy diagnosis, especially if the breeding date is unknown, it is recommended that a combination of progesterone, eCG and estrone sulfate assays be used. Alternative tests for pregnancy diagnosis have come and gone over the years.

8.6 *Techniques used in pregnancy assay*

Since pregnancy can be detected on the basis of pregnancy associated hormone, a number of techniques are available (Table.1).Depending upon the stage of gestation, type of hormone to be evaluated and lab facility available, either of them can be used for correct diagnosis of pregnancy status. Comparative studies done for evaluating the sensitivity of different tests based on eCG, have revealed that ELISA is very sensitive, as it could detect as low as 10 mIUeCG while in Hemagglutination inhibition (HI) assay and bioassay, the response was observed with higher quantity of PMSG contents (Relan, 2001).

Pregnancy test	Principle	Stage of pregnancy	Sample	Technique
Progesterone	Predicts luteal activity	Implantation	Serum/ milk	Radio immunoassay (RIA)/ Enzyme linked immunosorbent assay (ELISA)
Estronesulphate	Determines feto - placental function	Post-implantation	Serum/ milk/ urine	RIA/ELISA/ Chemical test
Equine chorionic gonadotropin eCG (PMSG)	Determines placental activity	Post-implantation	Serum	ELISA, Bioassay, Hemagglutination inhibition (HI) test, Radio -receptor assay, Direct latex agglutination test
Pregnancy associated antigens	Identifies antigens specific to pregnancy	Post-implantation	Serum	Hemagglutination inhibition (HI)

Table. 1. Techniques that can be used for pregnancy diagnosis in equines

अश्व प्रजनन संबंधी महत्वपूर्ण जानकारियां

मादा अश्वों में यौन परिपक्वता 3 से 3.5 वर्ष में आती है। परिपक्वता पूर्व प्रजनन से मादा अश्व में प्रसव के समय परेशानी, जननी या बच्चे की मृत्यु और स्वाथ्य बिगडना जैसे नुकसान हो सकते है।

मादा अश्व ऋतुकालिक प्रजनक होते हैं जो साल के लंबे दिन के अवधि में ही प्रजनन करते है। उत्तरी गोलार्द्ध के प्रदेशों में ज्यादातर मादा अश्वों में प्रजनन का समय मार्च से लेकर अक्टूबर तक का होता है।

प्रजनन ऋतु में मादा अश्वों में एक नियमित समय (21-25 दिन) पर मद चक्र की आवृति होती है जिसके दौरान मादा अश्व 4 से 8 दिन (औसतन 6 दिन) मद में होती है।

मद के समाप्त होने से 1-2 दिन (24-36 घंटे) पहले मादा अश्व अंडाशय में बने डिम्ब से अंडा निस्सारित करती है। अंडे का जीवनकाल (निस्सारित होने के 12 घंटे तक) शुक्राणुओं के जीवनकाल (वीर्यस्खलन के 48-72 घंटे तक) के अपेक्षात बहुत कम होता है।

मादा अश्व का नर अश्व से प्रातिक मिलान का उचित समय मद के 5 वें और/या 7 वें दिन होता है।

मादा अश्व में त्रिम गर्भधान का सही समय डिम्ब से अंडा निस्सारण के 12 घंटे पहले से 6 घंटे बाद तक उचित होता है। अंडा निस्सारण न होने पर त्रिम गर्भधान प्रति दिन एक के हिसाब से दो - तीन बार करना पड़ सकता है।

यदि गर्भाधान असफल रहा तो मादा अश्व में 19 से 24 दिन पर मद की आवृति होती है। गाभिन मादा अश्व भी कभी-कभी मद में आने के लक्षण दिखाती है जिसमें पशुचिकित्सक से जाँच करवा कर संदेह को स्पष्ट किया जा सकता है।

अश्वों में गर्भ की जाँच मशीन से 11-15 दिन में या बिना मशीन के गुदामार्ग से 30-45 दिन में संभव है। गर्भ के 35-120वें दिन में एकत्र किये खून के नमूने को प्रयोगशाला में भेज कर भी सीरम आधारित किट द्वारा गर्भ का पता कर सकते है।

अश्वों में गर्भकाल 310 से 370 (औसतन 340 दिन) होता है। गर्भकाल में होने वाले बीमारियों (टेटनस, ई.एच. वी., रेबीज, इंफ्लुएंजा इत्यादि) से बचाव के लिए नियमित समय पर टीकाकरण करायें!

प्रसव के 2-3 सप्ताह पहले थन के आकार में वृद्धि दिखाई देने लगता है और 6-48 घंटे पहले चुचुक पर गाढा पदार्थ जमने लगता है। प्रसव के तुरंत बाद से 12 घंटे के अंदर बच्चे को खीस पिलाना आवश्यक है!

ब्याने के 9-15 दिन बाद मादा अश्व मद में आती है जिसको प्रसव उपरांत पहली गर्मी (स्नशकृद्य मद्रकृह्ल) कहते हैं। यदि प्रसव सामान्य हुआ हो और जननी स्वस्थ हो तो ही स्नशकृद्य मद्रकृह्ल में गर्भाधारण के प्रयास किये जाने चाहिए