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Reproductive Physiology and Assisted Reproductive Techniques in Mithun

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ABSTRACT

Mithun is a unique bovine species that lives at an altitude ranging from 300 to 3000 m MSL. The physiology of reproduction in mithun is not very similar with cattle and buffalo. Therefore, in this review, we elaborate in detail about the reproduction profile and different conservation strategies for propagating the mithun germplasm. Mithuns are shy breeders and usually seasonal influences were reported on their reproductive pattern. For artificial insemination (AI), semen can be collected in mithun, processed and preserved. A.I. using refrigerated chilled and cryopreserved results pregnancies in mithuns. Other assisted reproductive technologies such as estrus synchronization, multiple ovulations and embryo transfer technology (MOET) and cryopreservation of embryo has been assessed and implemented at farm level with success rate. Understanding of the normal reproductive physiology in mithun will help to draw suitable breeding and conservation plans for implementation to propagate this unique species.

1. Introduction

Mithun is a magnificent domesticated bovine species available in South/Southeast Asia especially in India, China, Bangladesh, Myanmar, Bhutan and Nepal with highest population in India (3.9 Lakh; livestock census, 2019). In India, it is available in North Eastern hilly regions especially Arunachal Pradesh (350154), Nagaland (23123), Manipur (9059) and Mizoram (3957). It is commonly believed that mithun originated more than 8000 years ago from the wild Indian Gaur, *Bos gaurus* (Simoons, 1984 and Blench, 1999). Mithun is well adapted anatomically and physiologically to altitudes ranging from 300 to 3000mMSL. Mithun is living as small groups containing one adult male and several females and juveniles in the jungle. Mithun is meat animal in North eastern hill (NEH) region of India; however, it skin is very thick and is suitable for preparation of leather items. Even though mithun is not yet declared as an endangered species; however, it has been subjected to severe non-cyclic population fluctuations on a local or national or regional basis. Moreover, the mithun population is decreasing gradually due to the local unavailability of certified breeding bulls, lack of suitable breeding and feeding management, the increase of inbreeding practices and decline in the grazing land in mithun rearing regions. Knowledge on various aspects of reproduction and its interaction with environmental factors will help to assess the status of mithun reproduction and to improve the fertility of the animal; thus improve the socio-economic status of the local tribal populations.

Reproduction in females

Puberty and sexual maturity

Mithun cows present a high reproductive efficiency, as indicated by the reported calving-related traits (calving interval: 402.85 ± 3.04 days, gestation length: 296.25 ± 0.77 days), which allow to obtain one calf in a year (Faruque et al., 2015). Mithun is a polyestrous animal. Females become sexually mature at 2–3 years of age. The productive life ranges from 16 to 18 years. Physiological mechanism that controls puberty by changing interplay between hormones secreted from the hypothalamus, the anterior pituitary and the gonads lead to a transition from sexual quiescence to sexual function (Hull and Harvey, 2001). Among these complex endocrine interaction, growth hormone (GH) acts as a common hormonal link (Hull and Harvey, 2001). Study demonstrated that with increasing age and body weight (BW), growth hormone (GH) and GH per 100 kg BW both decreased and the animal with higher plasma GH and GH per

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100 kg BW showed higher growth rates in mithuns (Mondal et al. 2007). There was a higher LH concentration with higher pulsatility and greater amplitude in prepubertal mithuns than exhibited in growing mithuns (Mondal et al. 2005c). Aincreasing trend of plasma progesterone and LH concentrations has been recorded during the process of onset of puberty in mithun. Plasma GH levels were doubled during pubertal process. The optimum LH pulse frequency and amplitude required for onset of puberty in mithun were ‡9 pulses/24 h and ‡1.6 ng/ml, respectively (Mondal et al. 2005c)

Estrus cycle

Mean length of estrous cycle is 21.2 ± 0.3 days (19–24 days) and average duration is 12.6 ± 1.34 h. Mounting, congestion of the vaginal mucosa and swelling of the vulva are the common estrus signs. Other estrous signs such as mucus discharge, restlessness, tail raising, frequent urination and loss of appetite are less pronounced in mithun cows and length of estrus is longer in primiparous than in multiparous mithun cows. Sniffing of the vulva by bulls and standing to be mounted by bulls/other herd mates was recorded in 91.30% cases. The increased frequency of urination was observed in 82.61% of the female in estrus. Only 65.22% of Mithun females in estrus displayed restlessness. Bellowing was exhibited in 56.52% Mithun female (Hafez and Hafez, 1993). The homosexual behavior during estrus is less prominent in Mithun compared to cows. Seeking for the company of other animals (looking anxiously outside) was found in 56.52% of the Mithun females in estrus. Licking of the body of other animals during estrus was observed in 47.83%, but mounting herd mates during estrus was observed in only 26.09% of females. Reduced food intake or loss of appetite was observed in only 8.70% Mithun females in estrus (Mondal et al., 2006).

During estrus, the vaginal mucosa of Mithun females was recorded as reddish pink (pronounced), pink (moderate), and pinkish (slight) in 34.78, 43.48, and 21.74%, respectively. Edema of vulva was recorded in 82.61% of female Mithuns in estrus, of which pronounced edema and slight edema were recorded in 21.74 and 60.87% of estrus females, respectively. Discharge of the vaginal mucus does not always occur spontaneously in Mithun females in estrus, and has only been reported in 78.26% of the females. In 39.13% animals, the mucus was not discharged spontaneously until rectal palpation was carried out. The color of the vaginal mucus varied between transparent (61.11%), steel bluish (22.22%), and whitish (16.67%). Also, the consistency of the vaginal mucus varied from thin (55.56%) to thick (44.44%). The occurrence of foam in the mouth was observed in 30.43% animals (Samad, 1996).

Endocrine changes associated with estrous cycle

The emergence of the first follicular wave has been proposed to occur just after ovulation, while the follicle stimulating hormone (FSH) values are still elevated and the progesterone in low values. The emergence of the second follicular wave would occur approximately at day 14 of the cycle, in association with the raising FSH concentrations that will originate the first FSH peak, along with increasing luteinizing hormone (LH) and estradiol (E2) concentrations. The functional capacity of the corpus luteum (CL) starts decreasing from day 11 onward and circulating progesterone (P4) concentration starts reducing. This decrease allows the rising of FSH and, around day 16, the selection of the dominant follicle from the second follicular wave. The deviation of ovulatory follicle was proposed to occur around day 18 of the cycle (Dhali et al., 2005). The circulatory concentration of E2 starts to decrease with the regression of subordinate follicles. During the late stage of cycle, low concentration of P4 stimulates the rise in LH which helps in the final maturation of ovulatory follicle and triggers ovulation (Dhali et al., 2005). E2 and total oestrogen profiles during the peri-oestrous period showed that the mean highest peak concentrations of E2 (27.29 ± 0.79 pg/ml) and total oestrogen (45.69 ± 2.32 pg/ml) occurred at 3.90 ± 2.27 and 3.89 ± 2.26 h prior to the onset of oestrus, respectively. Plasma progesterone concentration was basal (0.14 ± 0.001 ng/ml) during the peri-oestrous period. Plasma E2 and total oestrogen were found to increase from 6 days before oestrus to reach a peak level on the day of oestrus and decline thereafter to basal level on day 3 of the cycle. The plasma progesterone concentration was the lowest on the day of oestrus showing gradual increase to register a peak level on day 15 of the cycle (Mondal et al., 2007).

Pregnancy and parturition

Length of gestation period is longer in mithun than in European cattle (Scheurmann, 1975; Majid et al.,1995), however; it is shorter for mithun × cattle crossbred (281.7 ± 1.2 days) (Giasuddin et al., 2003). Birth weight of female calves is lower than male calves (Perumal et al., 2014). Similarly, mithun has highest birth rate in September, December and January and lowest rate was recorded in May and June (Perumal et al., 2014). Mithun has shorter postpartum anestrus in calving during autumn and monsoon seasons (87.3 and 94.2 days, respectively) as compared to those calving in summer or winter (158.7 and 174.7 days, respectively) (Giasuddin et al., 2003). Higher conception rate was obtained in natural service or artificial insemination when the service or insemination was done at middle of estrus within 21–30 h of heat (Giasuddin et al., 2003).

The external signs of pregnancy in Mithun are similar to those of cattle, namely the abdominal distension, mammary gland development, and fetal ballottement. The abdominal distension, due to the gradual accumulation of voluminous uterine contents, can be detected after 5 months, but it is prominent only in more advanced stages of pregnancy. The udder development is noticed from 6 months onwards in Mithun heifers, or in the last 1–5 weeks of pregnancy in the case of multiparous cows. The teats are engorged at 12 h prepartum. Edema and relaxation of the vulva, derived from the progressive relaxation of pelvic ligament, is noticeable in the last few weeks of gestation. Three to four days before parturition, the vulvar lips become increasingly droopy and flaccid, along with a marked shrinking of the croup. In 74% of pregnancies, the fetus is balloted or its movement observed through abdominal wall after 6 months of pregnancy (Bhattacharyya et al., 2006).

Pregnancy diagnosis is done as similar to other bovine species. Slip of fetal membranes, distension of the uterine horn and presence of CL in ovary are noticed at 6th week of gestation. Pregnant uterus and ovary sink into abdominal cavity after 3 months of pregnancy and are not be palpated after 3 months of pregnancy. Fremitus of uterine artery is differentiable after 4 months and is much prominent or forceful from 6 months of gestation onwards. Enlargement of the middle uterine artery is observed after 5 months of pregnancy. Pregnancies in right horn are more frequently observed (60%) (Bhattacharyya et al., 2006). In 74% of pregnancies, the fetus is balloted or its movement observed through abdominal wall after 6 months of pregnancy (Bhattacharyya et al., 2006). Most of the calving occurs during the night (Ahmed et al., 2001) and signs of impending parturition are restlessness, increased micturition at 2–5 min interval and increased walking in mithun. Perumal (2014a) demonstrated Seed germination inhibition test on pregnancy diagnosis in mithun cows. Mithun licks the calf immediately after birth and calf stands up within 22 min of birth and first sucks colostrum within 30 min of birth. Average birth weight is 21 kg (Mondal et al., 2001). Dam browses and grazes around her baby. Calf brows and play around her mother and feeds of milk 12–15 times in a day. After 1 week, she gradually returns to her normal and the calf can be found moving freely in the herd (Giasuddin et al., 2003).

Incidence of reproductive problems

Reproductive problems are rarely observed in mithun. However, cases such as anestrus, metritis, dystocia, placental retention, and placentophagy or postpartum anestrus have been reported in mithun (Perumal et al., 2013). Similarly, the most common reproductive disorders in semi-intensive mithun farm of Bangladesh include metritis (16.7%), irregular heat (25.0%), anestrus (8.3%), repeat breeder syndrome (8.3%), abortion (16.7%), cervicitis (25%), and calf mortality (24%) (Huque et al., 2001).

Fertility improvement with exogenous kisspeptin

Mithun heifers injected with exogenous kisspeptin-10 (1.32 µg/kg body weight) at an interval of 3-4 days till commencement of puberty showed higher transcripts of Kiss1 and Kiss1R genes (approximately 2 folds) in pubertal than post pubertal animals that clearly shows the association between the Kiss1/Kiss1R genes and onset of puberty. Different methods were attempted to solve this anestrus disorder in mithuns; one such method is administration of Kisspeptin. Exogenous kisspeptin administration caused significantly early resumption of cyclicity in treatment as compared to control (24.64±10.43 vs 66.56±14.66 days) and significantly increased kiss1 and GPR54 mRNA expression in treatment as compared to control on the day of estrus.

Reproduction in males

Scrotal circumference and testicular parameters such as testicular volume and weight and its relationship with age and semen production was studied in mithun bulls. As per the reports, the scrotal circumference, testicular weight and semen production and its quality were positively correlated and increased up to 6 years of age and then decreased. Moreover, higher testicular weight and circumference have higher quantity and better quality semen. The optimum circumference for selection of mithun bulls was reported to be 28-33 cm for breeding purpose (Perumal, 2014b). Seminal vesicles of mithun bulls are lobular in structure and the number of lobes vary from 3 to 5 in both right and left seminal vesicles. In mithun bulls, the penis is cylindrical in shape with a tapering end and hung in a pendulous sheath. Complete detachment of penis from prepuce occurred at an average age of 48.28±1.30 weeks when the average body weight is 184.43±2.72 kg. The earliest and latest age at complete release of penis in mithun bulls is 42 and 53 weeks of age, respectively (Bhattacharyya and Goswami, 2008).

In mithun, body weight, scrotal circumference and testicular weight and semen quality parameters increases with the advancing age. Sperm output per bull per unit of time is lower in bulls of 25 – 36 months of age and highest is observed at the age of 73 months and above in mithun (Perumal and Rajkhowa, 2013).

Mithun bulls follows some sort of seasonality on semen production although they are perennial breeders. Result revealed that the bulls in winter and spring had significantly higher FSH, LH, and testosterone, T4, IGF-1 and melatonin (MT) than that in summer whereas the bulls in summer had significantly higher cortisol and prolactin than those in winter and spring seasons. Similarly, FSH, LH, testosterone, T4, IGF-1 and MT were significantly higher in night than in day time collections whereas cortisol and prolactin were significantly ($p < 0.05$) higher in day than in night time collections in different seasons. Spring and winter have significantly greater beneficial effects than summer on reproduction and artificial breeding programs in mithun (Perumal et al., 2021).

Seminal attributes and reports on semen preservation

Mithun semen can be collected through trans-rectal massage method and artificial vagina method preserved in liquid as well as in ultra-low temperature (cryopreservation). An estrus mithun cow is required for semen collection in A.V. method. The semen collection by massage method is more or less similar to cattle but it is difficult to massage the reproductive organs in mithun bulls and it takes longer time for ejaculation of semen. The volume of semen is significantly higher in collection through AV method than rectal massage method of collection. In AV method, mean reaction and the ejaculation time are 27.92±3.63 and 113.83±6.65 sec, respectively. Concentration of semen is also higher in the semen collected through AV method. Percentage of intact acrosome is also higher in semen from AV method (92.97 ± 0.90 vs. 89.83 ± 0.99). The semen characteristics of mithun bulls are generally within the range for fertile domestic bulls, although ejaculate volume and number of sperm is lower than that of domestic bulls, commensurate with smaller testes in mithun bulls (Bhattacharya et al., 2004).

Recently, comparative study on fresh and frozen-thawed semen quality and fertility of semen collected by artificial vagina and electro-ejaculation method in mithun were documented by Nadaf et al., (2021). Fresh and frozen-thawed semen quality parameters and motility and velocity profiles recorded by computer-assisted sperm analyser (CASA) were significantly ($p < 0.05$) lower in electro-ejaculation than the AV method. The conception rates at day 35–45 post insemination were non-significantly higher ($p > 0.05$) in the AV method. Thus, AV method has better semen quality; however, electro-ejaculation has the potential for semen collection from free-range mithun bulls to incorporate in assisted reproductive technology procedures (Khan et al., 2017)

Baruah et al., (2016) reported that additional of 5% glycerol is suitable and is optimum to preserve the mithun sperm in liquid nitrogen. Inclusion of 5% glycerol in the tris egg yolk treated extender had significantly improved the percentage of progressive motile, live and acrosome intact sperm and minimized sperm with morphological abnormality both at stage freezing or thawing, whereas the lowest sperm quality was observed in the extender containing 6 and 7% glycerol. Thus, the cryopreservation of semen in mithun with 5% glycerol with split dosage was standardized.

Assisted reproductive technologies

Artificial insemination

The method of Artificial Insemination is standardized in mithuns. Mithun semen can be successfully preserved at 4°C (liquid refrigerated semen), for approximately 2 days. It was also successfully cryopreserved in liquid nitrogen using a Tris-egg yolk-glycerol or citrate-egg yolk-glycerol extender with 5% glycerol concentration. Sperm quality can be improved (approximately 23–25% increase in progressive motility and count of live sperm with intact acrosome) by adding 5% glycerol in split doses instead of in a single dose. It was also observed that Tris-egg yolk-glycerol extender was better than citrate-egg yolk-glycerol's for cryopreservation of mithun semen. Inseminations with both frozen and liquid semen successfully produced calves through AI both at farm and field levels (Mondal et al., 2014)

Estrus synchronization

Protocols for estrus synchronization and timed AI has been developed for the mithun, including those using prostaglandin F2α (PGF2α) alone, the Ovsynch protocol (GnRH-PGF2α-GnRH), and progesterone-based controlled intra-vaginal drug releasing device (CIDR) (Mondal et al., 2014). In the synchronization protocol based on prostaglandins, two injections of PGF2α 11-day apart are given to cyclic mithun cows. Estrus signs following the injection of PGF2α indicated that mithun cows responded to this treatment and insemination can be done. The time from onset of estrus to ovulation is 27.7 ± 0.61 h, with a range of 26–31 h, in PGF2α-treated compared with 26.9 ± 0.31 h, ranging from 26–29 h in untreated cows (Mondal et al., 2014). The Ovsynch protocol was proved useful for estrus synchronization of cyclic mithun cows irrespective of the day of the estrous cycle at the beginning of the treatment. This protocol had a very good response from mithun cows; a 75% conception rate was obtained when using this protocol (Mondal et al., 2014). CIDR is a very useful approach to synchronization of estrus in cyclic as well as postpartum anestrus mithun cows. Very interestingly, the use of CIDR at 45–50 days postpartum induced estrus at 53–58 days, when the uterine involution was completed. In natural conditions, mithun cows exhibit the first postpartum estrus at around 102 ± 19.6 days postpartum. CIDR is therefore useful and advantageous for the expression of clear physical and behavioral signs of heat, which facilitates heat detection, as well as a higher productive lifespan of at least 50 days (Mondal et al., 2014).

Multiple ovulation and embryo transfer (MOET)

MOET is not only to be used in genetic improvement of mithun, i.e., in situ conservation, but also used in ex situ conservation of mithun. The superovulation/multiple ovulation and embryo transfer technology (ETT) has been successfully standardized for the mithun species. The first mithun calf, BHARAT, obtained through multiple ovulation and embryo transfer technology born on March 27, 2012, and the second calf (PRITHVI), born on May 11, 2012, at ICAR-National Research Centre on Mithun (Medziphema, Nagaland, India). Cryopreservation of mithun embryos has also been standardized. MOHAN, the first mithun calf, was born in May 12, 2012 from transfer of a 100-day-old cryopreserved embryo, also at ICAR-National Research Centre on Mithun. The standardization of the embryo transfer protocol in mithun will help in the conservation and propagation of quality germplasm in all the mithun-inhabited areas of NEH region (Mondal et al., 2014).

Conservation strategies

Keeping in view the dwindling population of mithun over the years, it is of great priority for the mithun inhabited states to conserve and propagate quality mithun germplasm at faster rate to stabilize its population. There are three ways for the conservation of mithun genetic resources: i) through cryopreservation of genetic material like ova, embryos or semen; ii) preservation of genetic information as DNA; and iii) conservation of live population (*in situ* conservation).

The need for parallel conservation of mithun genetic resources along with live animal conservation, as a raw material for future breeding programmes, should be recognized and has become an important issue in planning of mithun husbandry. Conservation is of particular concern in the mithun inhibited regions where there is effort for agricultural change, thereby the risk of gradual replacement of indigenous stocks and farming methods by new techniques. These areas, where climatic extremes and particular parasitic conditions may result in genetically modified and unique local stocks which are able to survive under extreme conditions, need to be given proper attention. Such conservation efforts are particularly important in the light of predicted global climate change, and the ability of microbial and insect parasites to evolve and adapt to modern chemical control methods. *Ex situ* preservation involves the conservation of mithun in a situation removed from their normal habitat. It is used to refer to the collection and freezing in liquid nitrogen of animal genetic resources in the form of living semen, ova or embryos. It may also be the preservation of DNA segments in frozen blood or other tissues. Finally, it may refer to captive breeding or other situations far removed from their indigenous environment. *In situ* conservation is the maintenance of live populations of animals in their adaptive environment or as close to it as is practically possible. For domestic species the conservation of live animals is normally taken to be synonymous with *in situ* conservation

2. Summary and conclusion

Various researches were conducted on morphology, structures and functions of the reproductive organs in mithun in comparison with cattle. Season has significant influence on reproductive pattern, scrotal and testicular biometrics, endocrinological profiles, semen quality profiles and fertility rate in mithun, although mithun is a perennial breeder and breed throughout the year. Winter and spring season has beneficial effect on production and reproduction traits in mithun. Semen can be preserved successfully in liquid and cryopreserved state and artificial insemination results higher calving rate. Semen quality in both liquid and cryopreservation can be improved with different methods, like inclusion of additives in extender and supplementation of antioxidants in dilutor etc. Different assisted reproductive technologies were successfully implemented with success rate in mithun. The reproductive technologies can be successfully implemented to conserve the declining mithun population in the country.

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