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Effect of different biostimulation methods on endocrinological profiles of mithun bulls

P PERUMAL[1]

ICAR-National Research Centre on Mithun, Medziphema, Nagaland 797 106 India

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ABSTRACT

A study was conducted to assess the effect of biostimulation methods on endocrinological profiles of mithun bulls. Adult male (6, 4–5 year old) with good body condition score (5–6) from herd of mithun farm of the institute were selected for the present study. The biostimulation methods were grouped into 9 groups and each group consisted of 6 animals, viz. Gr 1 (without exposure of female), Gr 2 (exposure of urine of non-estrus female), Gr 3 (exposure of urine of estrus female), Gr 4 (exposure of dung of non-estrus female), Gr 5 (exposure of dung of estrus female), Gr 6 (exposure of sweat of non-estrus female), Gr 7 (exposure of sweat of estrus female), Gr 8 (exposure of non-estrus female) and Gr 9 (exposure of estrus female). Exposure to each group was done with the interval of 1 month. The experiment was replicated 3 times. Blood samples were collected 15 min after exposure to the different methods of biostimulation for estimation of testosterone, thyroxine (T4) and cortisol, and at 30 min for follicle stimulating hormone (FSH) and luteinizing hormone (LH). Hormone profiles revealed a significant difference among the different methods of biostimulation in mithun bulls. The hormone profiles were significantly higher in adult mithun bulls that were exposed to estrus female followed by exposed to urine, dung, sweat of estrus female and lowest was observed in secretions of non-estrus animal groups. Through this investigation, a suitable model was developed to improve the endocrinological profiles by use of biostimulation techniques in mithun bulls which inturn will indirectly support will to higher semen production and its quality for artificial breeding programme.

Key words: Biostimulation, Dung, Endocrinological profiles, Estrus cow, Mithun breeding bull, Sweat, Urine

Mithun (*Bos frontalis*), a unique domesticated rare bovine species, is reared in semi-intensive or free-range extensive system in the North Eastern Hill (NEH) region of Indian sub-continent especially in Arunachal Pradesh, Mizoram, Manipur and Nagaland. The mithun is not yet endangered bovine species but is suffering from severe non-cyclical population fluctuations on a local/regional basis due to various reasons including lack of proper breeding management and breeding bulls. Collection method (Bhattacharyya *et al*. 2009), season (Perumal *et al*. 2015), vaccination (Perumal *et al*. 2013), breed (Rajoriya *et al*. 2013), pathological conditions (Perumal *et al*. 2016), and scrotal and testicular parameters (Perumal and Rajkhowa 2013), etc. significantly affect semen production and its quality, freezability and fertility of sperm and breeding bulls and endocrinological profiles which consequently affect the one such factor affect the reproductive status and fertility in mithun bulls (Perumal *et al.* 2017). It is difficult to detect estrous in mithun cows through visual observations. Bellowing is not generally observed in mithun during estrus. Similarly, mithun bull is shy in nature and it does not mount the cows that are not in estrous (heat) and does not mount and ejaculate the semen in artificial vagina. However, semen ejaculates were collected by artificial vagina method has significantly higher production and quality as compared to those collected through trans-rectal massage method (Bhattacharyya *et al*. 2009). Mandal *et al*. (2010) reported that semen was collected from mithun bulls by using the fresh or refrigerated stored estrus urine of mithun. Reports are available in mithun species on hormone profiles in different age groups at different seasons (Perumal *et al*. 2017). However, there are reports on different methods of biostimulation on sexual libido and ejaculation in different animal species and biostimulation is due to presence biomolecules in the secretion of external body fluids. Most chemical substances or signals are highly species-specific (copulin from the vagina of the rhesus monkey, Michael 1973; valeric acid or a mixture of fatty acids produced by oestrous felids, Bland 1979 and frontalin in Asian elephants, Rasmussen and Greenwood 2003). There is paucity of information on the effect of different biostimulation methods on endocrinological profiles of mithun bulls. Therefore, the present study was designed to assess the effect of different methods of biostimulation on endocrinological profiles of adult breeding mithun bulls to

Present address: [1]Scientist (perumalponraj@gmail.com), Division of Animal Science, ICAR-Central Island Agricultural Research Institute, Port Blair, Andaman and Nicobar Islands, India.

select the suitable method(s) for semen collection by using the artificial vagina method.

MATERIALS AND METHODS

Experimental animals: Apparently healthy adult mithun bulls (6) of approximately 4 to 6 year of age were selected from mithun breeding farm of the institute. The average body weight of the bulls at 4 year-old was 505 kg (495 to 510 kg), which increased to 530 kg (520 to 540 kg) in 6 year-old with good body condition (score 5–6), maintained under homogeneous feeding, housing, lighting and managemental conditions. Each experimental animal was daily offered *ad lib.* drinking water, 30 kg mixed jungle forages (18.4% dry matter and 10.2% crude protein) and 4 kg concentrates (87.1% dry matter and 14.5% crude protein), fortified with mineral mixture and salt.

Experimental groups: The biostimulation methods were divided into 9 groups and each group consisted of 6 animals, viz. Gr 1 (without exposure of female), Gr 2 (exposure of urine of non-estrus female), Gr 3 (exposure of urine of estrus female), Gr 4 (exposure of dung of non-estrus female), Gr 5 (exposure of dung of estrus female), Gr 6 (exposure of sweat of non-estrus female), Gr 7 (exposure of sweat of estrus female), Gr 8 (exposure of non-estrus female) and Gr 9 (exposure of estrus female). Exposure to each group was done with the interval of 1 month. The experiment was replicated 3 times. The estrus status of cows was detected by different behavioural and physical signs of estrus like standing heat, mounting behaviour, sniffing the vagina of another cow or resting chin on another cow (van Eerdenburg *et al*. 1996). Physical signs such as hyperaemia of vulvar mucous membrane, swelling of vulva, genital mucus through vulva on rectal palpation and presence of foam in mouth were observed. The rectal examination was performed to confirm the status of heat of the animals that expressed one or more symptoms or signs of heat. Moreover, bull parading was conducted every day in the morning and evening to find out the estrus animal. The cows with intensive estrus were captured and placed to collect the urine, sweat and dung separately; urine was collected after stimulation of the perineum and collected urine and dung was preserved at –80°C as different small allocates and used for the experimental period. Similarly, the same animals were used for collection of sweat with cotton towels. The collected estrus cow urine was sprinkled over the perineal region of mithun cow that were not in estrus and bulls were allowed to mount or react with the cow and blood samples were collected. Similar method was applied for dung also. The whole experiment was conducted in the completely confined animal shed with proper precaution. The experimental shed was isolated and kept away from the main animal sheds and not exposed to any animals or their movement around the shed. The bulls were placed and treated separately in the individual shed with no contact with other bulls in the experiment. All the experimental animals were treated or tested in the uniform method with same animal attendant and changing of clothes of animal attendant was mandatory before entering each room during the whole experimental period. The experiment was replicated 3 times.

Blood collection and analysis: The blood samples were collected by venipuncture of jugular vein in heparin tubes (20 IU of heparin/ml of blood) from the experimental mithun bulls 15 and 30 min after exposure to the different methods of biostimulation. The blood samples were centrifuged at 1,200 × g for 15 min at 4°C. The plasma samples were separated rapidly, labelled properly and preserved at –80°C in deep freezer for further estimation of testosterone, T4, cortisol and FSH and LH with commercial available diagnostic kits (RIA and ELISA kits). Follicle stimulating hormone (analytical sensitivity: 0.1 mIU/ml; intra- and inter-assay coefficients of variation: 6.47 and 9.54%, respectively) and luteinizing hormone (analytical sensitivity: 13 nmol/l; intra- and inter-assay coefficients of variation: 5.15 and 8.31%, respectively) were estimated with commercially available bovine ELISA kits (MyBiosource, San Diego, CA, USA) by optical density (λ 450 nm) in 96-well clear polypropylene microplate using a MRC Microplate Reader (UT-2100C, Israel). Hormones such as testosterone (analytical sensitivity: 0.02 ng/ml; intra- and inter-assay coefficients of variation: 5.13 and 9.27%, respectively), cortisol (analytical sensitivity: 5 nM; intra- and inter-assay coefficients of variation: 5.78 and 9.66%, respectively) and thyroxine (analytical sensitivity: 10.63 nmol/l; intra- and inter-assay coefficients of variation: 5.33 and 9.79%, respectively) were estimated by RIA based diagnostic kits (Immunotech, France) by using a gamma counter (PC-RIA MAS; Stretec, Germany).

Statistical analysis: The data were analysed statistically with the standard protocols (Snedecor and Cochran 1994). Analysis of variance was done with a generalized linear model (SAS Version 9.3; SAS Institute, Inc., Cary, NC) and using Student-Newman-Kuels (SNK) multiple range test. The data used in the study were tested for normality before analysis using Shapiro Wilk statistics. Differences with values of $P<0.05$ were considered to be statistically significant.

RESULTS AND DISCUSSION

Endocrinological profiles in the present study revealed a significant ($P<0.05$) difference among the methods of biostimulation in mithun bulls. The hormone profiles were significantly ($P<0.05$) higher in adult mithun bulls that were exposed to estrus female followed by exposed to urine, sweat, dung of estrus female and lowest was observed in bulls that were exposed to non-estrus females and its secretions. Highest effect was observed in exposure group of estrus cow followed by exposure of urine, sweat and dung of estrus female. Through this investigation, a suitable model was developed to improve the semen production and its quality by use of biostimulation technique by increasing the secretion of hormones in adult mithun bulls.

Sexual behaviour and libido in male is partially regulated and controlled by the hypothalamic-pituitary-gonadal axis.

Gonadotropin releasing hormone (GnRH) released from hypothalamus triggers the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH), which in-turn stimulates release of testosterone from the testes and enhance the spermatogenesis (Neave 2008, Nyby 2008). Moreover, changes in the episodic secretion of GnRH regulate the secretion of the gonadotrophins, LH and FSH in the prepubertal bull and thus, ultimately regulate testicular function for production of androgen as well as spermatogenesis (Courot 1978, Rodriguez and Wise 1989). It is well documented and reported that amplitude and frequency of LH pulses alter with the reproductive state of male animals (Ellis and Desjardins 1982) and there is a close temporal inter-relationship between these two hormones in bovine bull (McCarthy *et al.* 1979). In the present study, concentration of testosterone (Fig. 1), FSH (Fig. 1), LH (Fig. 1), thyroxine (Fig. 2) and cortisol (Fig. 2) were significantly (P<0.05) higher in bulls exposed to estrus mithun cows followed by its urine, sweat and dung, whereas significantly (P<0.05) lower values were observed in bulls that were exposed to non-estrus mithun cows and its body fluids and secretions. Male animals reflexively release testosterone when they smell (anticipatory releases) or mate (ejaculatory release) with a novel receptive female (Nyby 2008, Bonilla-Jaime *et al.* 2006). Variations in endocrinological profiles in mithun bulls is due to neuroendocrine and neurochemical mechanisms that occur on exposure to receptive female or its secretion (Naumenko *et al.* 1991, 1992). The female pheromones increase plasma testosterone and other hormones in male distantly (Amstislavskaya *et al.* 2004, Naumenko *et al.* 1992); intensity of enhancement varies with breeds or strains (Naumenko *et al.* 1983). Chemical signals are reliable indicators of an individual's reproductive condition because they are closely linked to physiological as well as endocrinological changes associated with reproduction. They are probably honest signals that convey to the receiver, the reproductive state of the sender (Gittleman 1989). Most chemical signals are highly species-specific (copulin from the vagina of the rhesus monkey, Michael (1973); valeric acid or a mixture of fatty acids produced by oestrous felids, Bland (1979); and frontalin in Asian elephants, Rasmussen and Greenwood (2003)). Thus, the importance of chemical signals has increasingly been recognized in reproductive biology to understand reproductive state and performance of individuals through analysis of species-specific behavioural cues (Berger 1992). Testosterone level increased to maximum 20 min after the introduction of receptive female and then over half-an-hour, decreased to a control level and the behavioural reaction started immediately and remained at a high level after 1 h (Pfaus *et al.* 2001), whereas, testosterone concentration increased in 20–40 min (Naumenko *et al.* 1983) after exposure of female odour which can be considered as a delayed response and response to the female odour is innate and species-specific. In the present study, there was variation in the concentration of the hormones in different experimental bulls and also in the same group. Different hormonal responses in males to the presence of a receptive female and its secretion could be due to different dynamics of blood testosterone changes conditioned by a great number of physiological mechanisms.

Pheromones in cows or its secretions are responsible to induce secretion of hormones through vomeronasal organ (VNO). In other mammals, stimulation of VNO with pheromones activates hypothalamic and limbic structures and results in changes of social and sexual behaviour and modulation of neuroendocrine reflexes and ultimately variation in the endocrinological profiles. It was reported that social stimuli such as the sight, sound and smell of a female in estrous can induce the endocrine changes in males (rhesus monkeys, Bernstein *et al.* 1977; domestic sheep, Gonzales *et al.* 1988; grey lag goose, Kotrschal *et al.* 2000) and similar result was observed in mithun in present study.

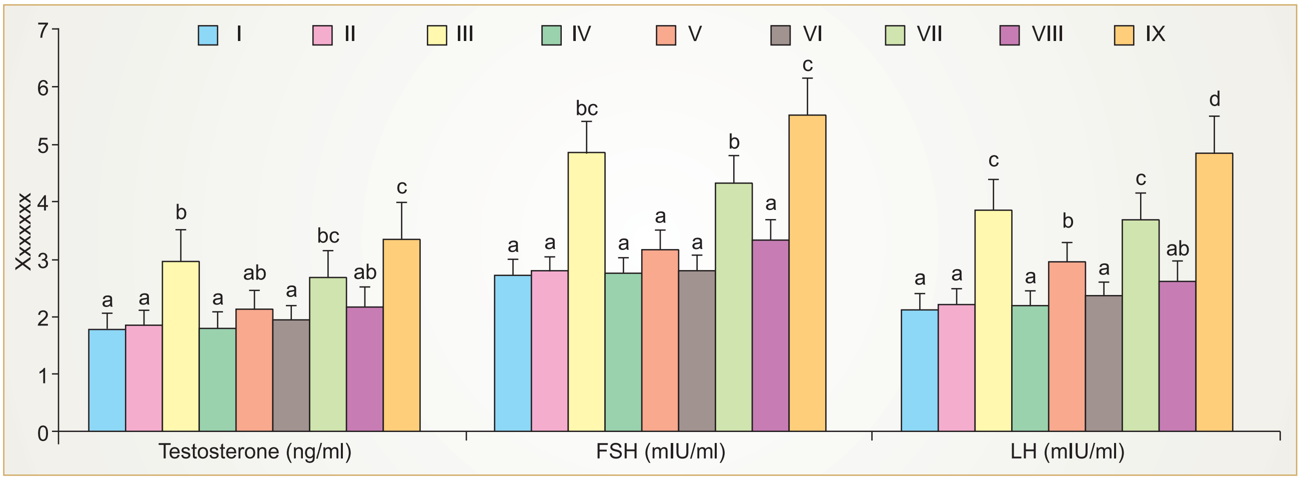

Fig. 1. Biostimulation on testosterone, follicle stimulating hormone (FSH) and luteinizing hormone (LH) of adult mithun bulls (*indicates differ significantly, p<0.05; n=6 for each group). Gr I, Without exposure of female; Gr II, exposure of urine of non-estrus female; Gr III, exposure of urine of estrus female; Gr IV, exposure of dung of non-estrus female; Gr V, exposure of dung of estrus female; Gr VI, exposure of sweat of non-estrus female; Gr VII, exposure of sweat of estrus female; Gr VIII, exposure of non-estrus female and Gr IX, exposure of estrus female.

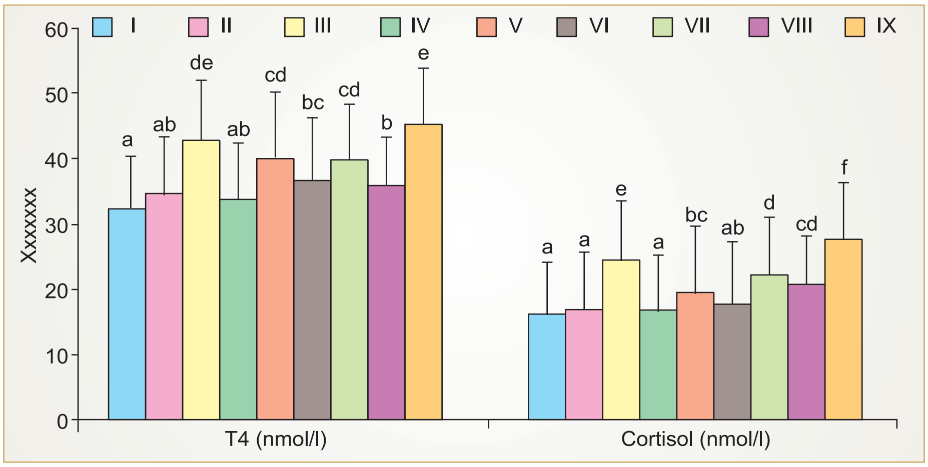

Fig. 2. Biostimulation on thyroxine (T4) and cortisol of adult mithun bulls (*indicates differ significantly, $p<0.05$; n=6 for each group). Gr I, Without exposure of female; Gr II, exposure of urine of non-estrus female; Gr III, exposure of urine of estrus female; Gr IV, exposure of dung of non-estrus female; Gr V, exposure of dung of estrus female; Gr VI, exposure of sweat of non-estrus female; Gr VII, exposure of sweat of estrus female; Gr VIII, exposure of non-estrus female and Gr IX, exposure of estrus female.

Males of a variety of species, have repeatedly been shown to respond to female stimuli by triggering an increase in LH immediately followed by a rise in plasma testosterone (Clancy *et al*. 1988). Similar condition was also observed in the present study. Rapid testosterone release occurs in at least two reproductive situations, the first upon initial exposure to a female and also following ejaculation should the interaction lead to mating. Generally, hormone levels rise within 10 min of encountering a female, peak within 20–30 min and return to basal levels within 80 min (Richardson *et al*. 2004). Moreover, the female is not required for behavioural stimulation and female's urinary scent is enough to trigger an identical testosterone secretion (Maruniak and Bronson 1976) and testosterone response is pheromonally driven (Wysocki *et al*. 1983, Coquelin *et al*. 1984). Clancy *et al*. (1988) found that while female urine elicited LH response in sexually naive male, the response was more robust when the animals were sampled again after they became sexually experienced. This indicated that the presence of the female herself is not necessary, as urinary pheromones alone are effective in triggering the testosterone response (Maruniak and Bronson 1976). Male also responds to urinary odours from females at any stage of the estrus cycle (Johnston and Bronson 1982). In rats, urinary odours of females in heat are highly attractive to intact male (Clancy *et al*. 1988). Males prefer the urine of estrus females to that of non-estrus females (Coquelin *et al*. 1984). Carr *et al*. (1965) reported that only males with heterosexual experience prefer odours from receptive females to odours of non-receptive females. However, when urine, feaces and vaginal discharges were exposed to the subjects, sexually naive males showed a clear preference for odours from estrus females. Similar result was observed in the present study. Borg *et al*. (1992) reported that in rams, the most consistent endocrine responses to estrus ewes are observed in sexually experienced males. Although the exact mechanism is unknown, it seems likely that males with sexual experience are able to associate mating behaviour with a female stimulus and produce a stronger or more specific LH and testosterone signal. It has been reported in several studies that bulls are able to discriminate between oestrous and non-oestrous odour (Paleologou 1977). Sankar and Archunan (2004) found that the duration of flehmen behaviour displayed by bulls was greater during exposure to oestrous samples of vaginal mucus, saliva, faeces and milk smeared on to the genital region of non-oestrous cows than during exposure to samples collected during other oestrous cycle stages. Among the substances tested, the response to oestrous vaginal mucus was significantly higher than for the other substances. Similarly in the present study, urine was most effective to trigger the endocrine changes followed by sweat and dung substances of estrus. Klemm *et al.* (1987) and Rivard and Klemm (1989) also presented their samples to bulls in a dish. The substances studied were vaginal mucus (Rivard and Klemm 1989, Klemm *et al.* 1987) and serum and vulval skin gland secretions (Rivard and Klemm 1989). They found that all 3 of these body fluids from cows in oestrus evoked a series of chained behaviours, which could be divided into 3 categories (Rivard and Klemm 1989). The attraction phase comprised initial responses such as head orientation toward the sample, sniffing the air, moving toward the sample, sniffing the sample at close range, salivation and urination. It was followed by the detection phase, which included behaviours such as licking, tongue manipulation, hyper-salivation, laboured breathing, flehmen and vocalisation. During the phase of sexual preparation, the bulls displayed penis protrusion, penile secretion and head butting and mounting behaviour. These observations were observed in the present study also. Kumar *et al.* (2000) analysed urine from cows in different stages of cyclicity and found that 1-iodo undecane and di-n-propyl phthalate were unique to the oestrous samples. Further evidence that 1-iodo undecane is oestrus-specific was provided by Sankar and Archunan (2008). When they compared volatile profiles from faeces of cows in different stages of the reproductive cycle, they found three compounds, viz. acetic acid, proprionic acid and 1-iodo undecane, that were specific for the oestrous phase. The dispersion of oestrus-specific compounds in the bovine body has been demonstrated previously in swabs from the vulva and fluids from the vagina, urine, milk and blood (Rivard and Klemm 1989, Kiddy *et al.* 1984). However, in the present study, the active compounds in the urine, dung or sweat in mithun were not estimated. It was concluded from the present study that highest effect on endocrinological profiles was observed in exposure of estrus cow followed by exposure of urine, dung and sweat of estrus female and lowest was in bulls exposed to non-estrus cows and its secretions.

Through this investigation, a suitable model was developed to improve the endocrinological profiles by use of biostimulation techniques in mithun which indirectly supports higher semen production and its quality for artificial breeding programme in mithun.

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