



Effect of biostimulation on sexual behaviour and semen production in Sahiwal bulls

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ABSTRACT

The study was planned to overcome the poor libido problem through biostimulation on eight healthy and adult Sahiwal breeding bulls not donating semen at Artificial Breeding Research Centre, ICAR-NDRI, Karnal, Haryana. The bulls were first taken as control and their sexual behaviour was recorded by CCTV video camera. The bulls were biostimulated by applying estrous specific mucus on the rump region of dummy prior to semen collection. Out of 8 bulls, 6 bulls (75%) started donating good quality semen after application of estrous specific mucus. The 6 bulls produced quality semen having optimum ejaculate volume, mass activity, initial progressive motility, concentration, pH, non eosinophilic spermatozoa count, total morphological abnormality, HOST, acrosomal integrity, SYBR-14PI and CMA3. There was no significant difference observed in semen quality and sexual behaviour between cervical mucus and synthetic nasal spray treated groups. All sexual behaviour parameters significantly improved in both the treatment group as compared to control. Thus biostimulation by cervical mucus is effective for quality semen production besides better sexual performance of bulls.

Key words: Biostimulation, Cervical mucus, Poor libido, Sahiwal bull, Synthetic nasal spray

One of the major reasons for disposal of indigenous breeding bulls is poor libido followed by poor semen freezability and quality (Khatun *et al.* 2013). Poor sexual behaviour decreases work efficiency of persons involved in semen collection (Le Danvic *et al.* 2015). Increasing demand of quality semen has renewed the emphasis on libido maintenance of bulls used in artificial insemination. The disgrace of shyness and sexual sluggishness attached with indigenous breeding bulls and their preference towards females in full oestrous for mounting is well known (Chenoweth and Steven 2014). Therefore, scientific and most effective methods of sexual preparation and stimulation are required to harvest optimum quality semen in dummy with AV and maintenance of bull's libido for quality semen production. Scientists worked on various aspects to improve libido of bulls by spray of estrous specific molecule in nasal cavity (Le Danvic *et al.* 2015). Therefore, to improve the libido and quality semen production of breeding bulls, an experiment was planned to study the efficacy of estrous mucous and synthetic pheromone on semen production performance of Sahiwal

bulls in frozen semen production centre.

MATERIALS AND METHODS

Healthy adult Sahiwal breeding bulls (8), 34 to 56 month-old, maintained at the Institute, were used and ration, vaccination, deworming and other herd-health programme was followed as per the farm schedule. Bulls were kept in individual bull pen 30' × 10' separated by solid partitions. Bulls were exercised once a week.

The bulls were scheduled for routine semen collection from 18 months of age, however, all the 8 bulls showed poor libido and did not donate semen in AV on teaser bull. In the experiment, more than 10 observations of sexual behaviours in semen collection yard by CCTV camera video recording was collected. The bull before treatment served as control and they had not donated semen in AV. In first biostimulation treatment, eight bulls were exposed to cervico-vaginal mucus collected from oestrus Sahiwal cow, by AI gun with blue sheath (IMV). The oestrous condition of cows was recognized based on the basis of visual observations, rectal palpation and examination of typical fern pattern of cervical mucus (Layek *et al.* 2013). The mucous was stored at -20°C. Before actual application of mucus on the rump region of the dummy just before semen collection, the mucus was brought to room temperature, defibrinised with the help of rotator and dissolved in distilled water in a spray bottle. After completion of first treatment

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with cervico-vaginal mucus, 2 week refractory period was provided to the bulls, during this period, no cervico-vaginal mucus was applied for semen collection. Commercially available synthetic nasal spray was used to stimulate sexual function and increase the reproductive capacity of the bull (Le Danvic *et al.* 2015). Synthetic nasal spray was applied on nostril of Sahiwal bulls about 10 min before semen collection. Semen was collected, 2 ejaculates/week, from the bulls using sterilized bovine artificial vagina over a male dummy bull. Soon after collection, each ejaculate was placed in a water bath at 30°C and various standard laboratory tests for semen were recorded.

Quality of the semen was assessed for ejaculate volume and microscopic tests such as mass activity (MA), individual progressive motility (IPM), sperm concentration (Improved Neubauer's chamber method), non-eosinophilic spermatozoa count (NESC), acrosomal integrity (AI), sperm abnormalities, using DIC phase contrast microscope with Tokoiheat thermal stage (Bhakat *et al.* 2016). The percentage of membrane intact spermatozoa was evaluated using hypo-osmotic swelling test using hypoosmotic solution (150 mOsm/l). For analysing live, acrosome intact and membrane intact spermatozoa, at least 200 sperms/smear were counted using tally counter. All semen evaluations were performed by same person throughout the study. SYBR-PI was used to determine membrane integrity and viability. Viable cells stained green with SYBR-14 whereas non viable cells stained red with propidium iodide observed under an epifluorescence microscope. Chromomycin A3 was used to detect protamine deficient DNA (Shivhare *et al.* 2015).

Sexual behaviour like sexual aggressiveness (SA), reaction time (RT), dismounting time (DT), total time taken in mounts (TTTM), penile erection score (PES), penile protrusion score (PPS), ejaculatory thrust score (ETS), mating ability score (MAS) and sexual behaviour score (SBS) on the basis of modified score card (Singh *et al.* 2014) was studied using CCTV video camera recording. Experimental procedures were approved by the Institutional Animal Ethics Committee (IAEC).

Statistical analysis: One way analysis of variance (ANOVA) was performed to compare sexual behaviour and

semen quality with respect to biostimulation intervention using Sigma Plot 11 software package. Prior to the analysis, proportionality data (initial progressive motility, non-eosinophilic spermatozoa count, HOST, acrosomal integrity, abnormality, libido score, mating ability score, sexual behaviour score data) were transformed using the arcsine transformation [$\text{asin}(\sqrt{\text{value}/100}) * 180/\text{PI}()$] (Snedecor and Cochran 1994). Comparison between different treatment groups was done by Tukey's multiple pair-wise comparison test.

RESULTS AND DISCUSSION

Semen quality: The overall least squares means±SE of ejaculate volume, mass activity, initial progressive motility, concentration, pH, non-eosinophilic spermatozoa count (NESC), morphological abnormality total (head, mid-piece, tail), HOST, acrosomal integrity, SYBR-14PI and CMA3 are presented in Table 1.

Similar results of mass activity, initial progressive motility, sperm concentration, NESC (Ray and Ghosh 2013), total abnormality, HOST (Ramachandran *et al.* 2006) in Sahiwal bulls; SYBR14-PI (Shivhare *et al.* 2015) in Murrah buffalo bulls and CMA3 (Rajak 2012) in KF bulls. However higher values from the present estimates were reported in ejaculate volume, morphological abnormality, sperm concentration, HOST (Ray and Ghosh 2013) in Sahiwal bulls. The variation in results may be due to genetical, age, season, geographical location, type of experiment, skill of the evaluator and management practices. The major purpose of evaluation of semen quality was to ensure that the bulls started donating quality semen or not. However, the values are within the acceptable range of semen quality for normal Sahiwal breeding bulls.

Sexual behaviour: The overall least squares means±SE of sexual aggressiveness (SA), reaction time (RT), dismounting time (DT), total time taken in mounts (TTTM), penile erection score (PES), penile protrusion score (PPS), ejaculatory thrust score (ETS), libido score (LS), mating ability score (MAS), sexual behaviour score (SBS) are presented Table 2.

Results were similar on sexual aggressiveness, dismounting time to earlier reports (Reddy and Sasikala

Table 1. Effect of biostimulation on semen quality parameters of Sahiwal breeding bulls

Group	N	EV (ml)	MA (0-5 scale)	IPM (%)	SCON (million/ ml)	pH	NESC (%)	HOST	AI	SYBR- 14	CMA3	Spermatozoa			Abnormalities Total
												Head	Mid- piece	Tail	
Cervical mucus	30	3.78± 0.33	2.81± 0.16	72.33± 2.56	1149.67± 65.5	6.80± 0.02	81.83± 1.64	62.43± 1.80	91.17± 0.90	65.73± 07	2.09± 0.1	2.03± 0.32	0.93± 0.11	4.3± 0.18	7.27± 0.40
Synthetic nasal spray	33	3.22± 0.30	2.97± 0.16	72.88± 3.29	1125.79± 43.86	6.95± 0.06	84.73± 2.71	65.42± 2.81	87.94± 1.79	68.33± 1.71	1.79± 0.1	2.55± 0.31	0.52± 0.10	2.91± 0.2	5.97± 0.38
Overall mean	63	3.49± 0.22	2.89± 0.11	72.62± 2.09	1137.16± 38.44	6.88± 0.03	83.35± 1.61	64.00± 1.70	89.48± 1.04	67.08± 0.97	1.93± 0.9	2.30± 0.22	0.71± 0.08	3.57± 0.2	6.59± 0.29

Means bearing different superscripts within same column differ significantly (^{ab}P<0.05, ^{AB}P<0.01); N, no. of observations; EV, ejaculate volume.

Table 2. Effect of biostimulation on sexual behaviour performance of Sahiwal breeding bull

Group	N	SA (1–4 scale)	RT (sec)	DT (sec)	TM (sec)	PES (0–3 scale)	PPS (0–2 Scale)	ETS (1–3 Scale)	LS (%)	MAS (%)	SBS (%)
Control	80	2.56 ^{A±} 0.06	332.19 ^{A±} 32.12	1.97 ^{A±} 0.12	334.06 ^{A±} 32.14	1.16 ^{A±} 0.10	0.76 ^{A±} 0.07	0.20 ^{A±} 0.06	25.77 ^{A±} 1.20	24.44 ^{A±} 2.28	25.10 ^{A±} 1.53
Cervical mucus	82	3.44 ^{B±} 0.06	207.23 ^{B±} 23.33	4.23 ^{B±} 0.25	211.44 ^{B±} 23.34	2.17 ^{B±} 0.12	1.13 ^{B±} 0.09	1.15 ^{A±} 0.15	36.51 ^{B±} 1.67	55.06 ^{B±} 4.21	45.78 ^{B±} 2.69
Synthetic nasal spray	77	3.45 ^{B±} 0.06	212.45 ^{B±} 25.85	4.23 ^{B±} 0.24	216.68 ^{B±} 25.87	2.19 ^{B±} 0.13	1.17 ^{B±} 0.10	1.16 ^{B±} 0.16	37.94 ^{B±} 1.85	55.91 ^{B±} 4.39	46.92 ^{B±} 2.88
Overall mean	239	3.15± 0.04	40.65± 15.92	3.66± 0.16	244.28± 15.90	1.84± 0.07	1.02± 0.05	0.83± 0.08	33.38± 0.98	45.08± 2.35	39.23± 1.54

Means bearing different superscripts within same column differ significantly (^aP<0.05, ^{AB}P<0.01); N, no. of observations; SA, sexual aggressiveness; RT, reaction time; DT, dismounting time; TM, total time taken in mounts; PES, penile erection score; PPS, penile protrusion score; ETS, ejaculatory thrust score; LS, libido score; MAS, mating ability score; SBS, sexual behaviour score.

2013) in crossbred bulls, but slightly higher on PES, PPS, ETS than that of Reddy and Sasikala (2013) in Sahiwal bulls.

Effect of biostimulation on semen production, semen quality and sexual behaviour: The worth fullness of the biostimulation intervention in the experiment reflected when Sahiwal breeding bulls who were not donating semen, started donating semen. Out of 8 bulls, 6 (75%) started donating semen after application of estrous specific mucous on the rump region of dummy prior to semen collection and similar results were obtained after application of commercially available synthetic nasal spray in 8 Sahiwal breeding bulls, but the 2 bulls which did not respond after application of estrous mucous, behaved similarly in case of synthetic nasal spray.

Effect of biostimulation on semen quality was analyzed and presented in Table 1. The bulls did not donate semen when acted as control, therefore no semen quality data is available to compare with treatment groups. However, there was no significant (P<0.05) difference found for ejaculate volume, mass activity, initial progressive motility (%), concentration (million/ml), pH, non-eosinophilic spermatozoa count (NESC, %), total morphological abnormality (head, mid-piece, tail), HOST (%), acrosomal integrity, SYBR-14PI and CMA3 between cervical mucus and synthetic nasal spray treated group.

Effects of biostimulation on sexual behaviour are presented in Table 2. All the sexual behaviour parameters SA, RT, DT, TTTM, PES, PPS, ETS, LS, MAS, SBS improved significantly (P<0.01) after cervical mucus and synthetic nasal spray application in Sahiwal breeding bulls as compared to control, but no significant difference was observed regarding sexual behaviour between cervical mucus and synthetic nasal spray treated group. The tactile stimulation behaviour in control group prior to mounting was 29.89% sniffing of dummy and 16.67% Flehmen reaction, respectively, whereas after cervical mucus and synthetic nasal spray application there was improvement in tactile stimulation behaviour specially sniffing of dummy (34.56 and 38.61%) and Flehmen reaction (25.81 and 19.80%).

The 6 bulls started donating semen after estrous mucous application and continued the production of semen during refractory period also; therefore, it is very difficult to depict the efficacy of synthetic nasal spray to some extent, although their performance related to sexual behaviour and semen quality improved as compared to control in both the treatment group. The 2 bulls could not be recovered either by estrous mucous application or synthetic nasal spray application. Therefore, regarding synthetic nasal spray one more study need to be carried out in a separate group of breeding bulls that were not donating semen, to judge the actual efficacy of synthetic nasal spray. Le Danvic *et al.* (2015) reported significant improvement of sexual behaviour (reaction time and ejaculation time) with increase in sperm quantity by 9% in some bulls having a weak libido but they were in semen production. Vyas *et al.* (2012) also reported that oestrus-related chemicals might also be useful as sexual stimulants for increasing the libido of unmotivated males.

The application of estrus cervical mucus and commercial synthetic nasal spray treatment improved the semen quality and sexual behaviour due to pheromones present in the compound applied for biostimulation. In the last two decades, researchers explored and increased basic knowledge of the chemistry of pheromones in cattle (Rajananarayanan and Archunan 2011). Basically pheromones act singly or in combination with other pheromones, which may regulate the reproductive and social behaviours through specific signals. Most of the work depicts on the importance of pheromone in female reproduction through influence on precopulatory behaviour, but use of pheromone is still not exploited fully for improvement of male reproduction. Males normally attracted towards females in estrous via pheromone secreted through urine, faeces and vagina (Sankar and Archunan 2008). Pheromonal signals work through volatile and non-volatile molecules for transmission of pheromonal information (Mucignat-Caretta *et al.* 2012) via main or accessory olfactory system. The pheromones act on vomeronasal organ of male during sniffing and it pass on the signal to accessory olfactory bulb (Mucignat-Caretta 2010), which is connected to the amygdale and

hypothalamus that generates pulses of gonadotropin-releasing hormone (Rekwot *et al.* 2001). GnRH acts on anterior pituitary stimulating release of FSH and LH. FSH in conjugation with testosterone acts on Sertoli cells of the seminiferous tubules of the testis and stimulate spermatogenesis. LH acts on Leydig cells of testis and stimulates production of testosterone, which is vital for spermatogenesis, male characteristics (Sekoni *et al.* 2010) and libido. Therefore, presence of estrus related odours during the pre-estrus and estrus period emphasizing the better efficacy of the estrous mucous in overall improvement of sexual behaviour and semen quality in our study. On the similar line, the effective role of urinary- volatile compounds in female buffalo during estrous, in enhancing the sperm quantity was also reported by Rajanarayanan and Archunan (2011). One of the major purposes of the semen quality evaluation was to ensure that the bulls start donating semen of acceptable quality. The bulls started donating semen of acceptable quality, which was further supported by advance *in-vitro* sperm function test like SYBR-14PI and CMA3.

The decrease in reaction time after cervical mucus and synthetic nasal spray intervention was also reported by Rodriguez-De Laraa *et al.* (2010), who informed the significant effect of biostimulation on reaction time in buck rabbits. Danvic *et al.* (2012) also reported the similar finding by 5 commercially available compounds (coumarin, 1, 2-dichloroethylene, squalene, 2-butanone, oleic acid) which, extracted from estrus cow urine, were tested alone or in combination for their biological activity when sprayed into bull's nasal cavities. Penile erection score (PES) and penile protrusion score (PPS) significantly improved in cervical mucus and synthetic nasal spray treated group in comparison to control, but there was no significant difference between cervical mucus and treatment group. The results are in consonance with the beneficial effect of biostimulation on PES, PPS and tactile stimulation by cervical mucus as reported by Sankar and Archunan (2004) in buffalo bulls. Karthikeyan and Archunan (2013) reported that a specific volatile synthetic nasal spray compound, 9-octadecenoic acid, was identified as estrus-specific in vaginal mucus, which elicit sexual stimulation in buffalo bulls. The improvement of overall sexual behaviour or libido in our study is in agreement with the observation reported by Rajanarayanan and Archunan (2011), who found that bulls exhibited repeated Flehmen behaviour, penile erection and mounting behaviour after sniffing estrus specific compounds (sex pheromone) present in urine of oestrus female. Improvement of sexual behaviour (reaction time and ejaculation time) after synthetic nasal spray application for weak libido bulls was reported by Le Danvic *et al.* (2015) is also supporting our finding and it can be used to improve productivity and easier handling of the weak libido bulls in semen collection centre.

The result emphasize the importance of experiment as superior quality bulls having good genetic worth started donating semen after estrous mucous application while

previously they were not donating semen. Efficacy of synthetic nasal spray need to be studied further separately as all the 6 bulls started donating semen after estrous mucous application and continued in semen production even during refractory period. All sexual behaviour parameters and semen quality improved significantly after estrous mucous and synthetic nasal spray application. Biostimulation can be utilized as an effective tool to optimize the quality semen production from elite indigenous bulls by reducing the disposal of poor libido bulls by applying estrous specific mucous to bring back them in quality semen production without involving much cost.

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