



## NOTE

Theriogenology

# The effect of false mount on quality of frozen-thawed semen in *Bos indicus* beef bulls

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**ABSTRACT.** This study evaluated the effect of false mounts (FM) on quality of frozen-thawed semen in beef bulls. Five mature Brahman bulls at the Standard Semen Production Center in Thailand were subjected to semen collection. Overall, 60 ejaculates were collected over a 12-week period of which 30 were collected without, and another 30 were collected using FM. A range of quality parameters of fresh, pre-frozen, and frozen-thawed semen were evaluated. Use of FM resulted in significant improvement of most parameters of fresh semen except progressive motility and sperm viability. The quality parameters of pre-frozen and frozen-thawed semen also significantly improved with FM. Use of FM significantly improved the viability of frozen-thawed semen, possibly indicating the protective effect of seminal plasma proteins.

**KEY WORDS:** beef bull, computer-assisted sperm analyzer (CASA), false mount, semen quality, sexual preparation

*J. Vet. Med. Sci.*

82(5): 673–677, 2020

doi: 10.1292/jvms.20-0025

Received: 13 January 2020

Accepted: 11 March 2020

Advanced Epub:

23 March 2020

In livestock, semen yield and quality are crucial determinants of reproductive success, and profitability of semen producing enterprises. In cattle, different methods of semen collection, including the use of artificial vagina, transrectal massage, and the application of electroejaculators, are used to maximize semen harvest from highly prized bulls. There are several physiological and environmental factors that influence the quantity and quality of semen produced by any bull. Factors affecting bull semen quality include temperature, humidity [13], bull age, frequency of semen collection [14], and duration of sexual rest [29]. Similarly, factors affecting semen quantity include testicular size [13] and frequency of semen collection [29]. In addition to the above factors, several studies have demonstrated that supplementary sexual preparation prior to semen collection could significantly improve bovine sperm harvest [17]. In both dairy and beef bulls, sexual preparation involving restraint and false mounts (FM) is widely known to maximize the sperm output per ejaculate [1, 6, 7, 9, 17]. Improvements in semen output by the use of sexual preparation has also been reported in other species such as boars [19]. In beef bulls, sexual preparation involves the application of three FM prior to the first ejaculation, and none prior to the second ejaculation [1, 7]. FM involve manual deviation of the bull's penis during a mount, in order to prevent intromission as well as ejaculation. This has been found to be an effective technique to enhance the sperm output [17] as a result of oxytocin release from the posterior pituitary resulting in enhanced smooth muscle contractions in the male reproductive tract.

In frozen semen production, cryopreservation process can cause damage to the bull sperm that results in lowered frozen-thawed semen quality, such as the sperm viability [22]. Generally, the process of semen cryopreservation decreases the percentage of viable sperm by approximately 50 percent [5]. Therefore, improving the quality of frozen-thawed semen is important especially in beef bulls. In Thailand, sexual preparation prior to bull semen collection is generally used in dairy bulls, however, it is not very common in beef bulls, especially in *Bos indicus* breeds. This is due to difficulty in handling bulls as well as due to increase labor cost and time for semen collection. Therefore, most of beef bull semen production centers perform the collections without any FM. Furthermore, the impact of FM in sexual preparation of bulls, on semen quality of frozen-thawed semen has not been thoroughly investigated. Therefore, the objective of this study was to evaluate the effect of FM on frozen-thawed semen quality of *Bos indicus* beef bulls in Thailand.

Semen was collected from five healthy and mature Brahman bulls aged between three to six years, body condition score of 3 (scale 0 to 5) [4], with body weight ranging from 700 to 950 kg, that were reared and handled uniformly at the Northeastern Region Standard Semen Production Center, Bureau of Biotechnology in Livestock Production, Department of Livestock Development, Thailand. The study was approved by the Charles Sturt University, Animal Care and Ethics Committee (Protocol

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number- A18075). Sixty semen samples were collected from five different bulls at one-week interval between 08:45 A.M. and 10:00 A.M. using artificial vagina (AV). Thirty bull semen samples (five bulls x six ejaculates, at one ejaculate per week) were collected without using FM from week one to six of the experiment for control series of semen collection. There was a transitional period of one week for training the bulls to do FM. Following this, another 30 semen samples (five bulls × six ejaculates, at one ejaculate per week) were collected from the same bulls with application of FM during week 8 to 13 of the experiment. The method in this study involved three FM without restraint prior to actual semen collection. The total time duration for three FM ranged between 15 to 25 min but remained almost the same for an individual bull during the experimental period. The wait time between FM also varied between bulls (3 to 4 min) but was very much the same for an individual bull over the experimental period. Manual deviation of penis was performed during each mount. Two castrated bulls at the same facility were used for mounting. All the semen samples were collected by the same collector as well as under same environmental conditions between November 2018 and February 2019. Average ambient temperature during the sampling time from week 1 to week 13 was 26.88°C (range 23.9 to 30.4°C) [32].

The stock extender solution used for semen processing was composed of Tris 30.28 g, citric acid 17 g, fructose 12.5 g, dissolved in 920 ml deionized water and glycerol 80 ml. The Tris-egg yolk extender was the stock extender solution with 1,000 mg streptomycin, 1,000,000 IU penicillin, and 250 ml egg yolk. Once collected, the fresh semen sample was transferred immediately to the laboratory, where it was kept in the water bath at 33°C for evaluating parameters that included: pH, volume, sperm concentration, gross motility, percentage of individual motility, sperm morphology, sperm viability, and functional integrity of sperm plasma membrane. Semen volume was measured in a pre-warmed test tube. The sperm concentration was determined using a spectrophotometer (Accucell photometer, IMV Technologies, L'Aigle, France), and semen pH was measured using pH indicator papers (Panpeha pH indicator strips, Sigma-Aldrich, Seelze, Germany). Sperm motility for fresh, pre-frozen and frozen-thawed semen samples were assessed via computer-assisted sperm analyzer (CASA-The IVOS II system driven by software version 14: Hamilton Thorne Inc., Beverly, MA, USA). The mean values for total motility (% of sperm cells that are moving irrespective of the direction) and progressive motility (% of sperm cells that are moving forward in a progressive manner) were calculated using CASA. A 7  $\mu$ l drop of the sample was placed on a warmed (37°C) CASA slide (Leja® slide: Leja Products BV, Nieuw-Vennep, The Netherlands). A minimum of five fields per sample were evaluated, and a minimum of one thousand spermatozoa per sample were counted. The percentage of sperm viability was evaluated using eosin-nigrosin staining method from Dott and Foster [11]. The semen sample (50  $\mu$ l) was mixed with a drop of eosin-nigrosin stain. The mixture (7  $\mu$ l) was smeared on a clean glass slide and air dried. The sperm viability evaluation was assessed by counting spermatozoa with a light microscope (Nikon, Tokyo, Japan) under 400 × magnification. The sperm cell with an unstained head was classified as live, whereas the sperm cell with red color staining was classified as dead. Two hundred spermatozoa per sample were evaluated to calculate the percentage of the live spermatozoa. The percentage of viable sperms in frozen-thawed samples was assessed by staining with SYBR-14/propidium iodide (PI) (Molecular Probes Inc., Eugene, OR, USA) as described by Garner and Johnson [15] with minor modifications. For staining, frozen-thawed semen samples (50  $\mu$ l) were mixed with 2.7  $\mu$ l SYBR-14 solution and 10  $\mu$ l of PI. The SYBR-14 solution was produced by diluting (1:100) in dimethyl sulfoxide (DMSO). After incubation at 37°C for 20 min, two hundred spermatozoa were assessed under a fluorescence microscope (Nikon Eclipse Ni series, Nikon) with 400× magnification. The nucleus of the live sperm cells showed bright green fluorescence from SYBR-14 staining, whereas the nucleus of dead sperm cells showed red fluorescence from PI staining. The results of sperm viability were recorded as the percentage of live sperm cells with bright green fluorescence (SYBR-14). Functional integrity of sperm plasma membrane was assessed using a hypo-osmotic swelling test (HOST) used by Revell and Mrode [27] with minor modifications. For fresh and diluted semen samples, spermatozoa were incubated at 37°C for 40 min, with 150 milli-osmole/kg of a hypo-osmotic solution consisting of 7.35 g of sodium citrate and 13.51 g of fructose in 1,000 ml distilled water. For frozen-thawed semen samples, spermatozoa were incubated at 37°C for 40 min, with 100 milli-osmole/kg of a hypo-osmotic solution consisting of 4.90 g of sodium citrate and 9 g of fructose in 1,000 ml distilled water. After the incubation time, 200  $\mu$ l of the semen-hypo-osmotic solution was fixed in 1,000  $\mu$ l of a hypo-osmotic solution plus 5% formaldehyde, for evaluation later. Sperm tail coiling was assessed by placing 20  $\mu$ l of well-mixed sample on a warm slide that was covered with a coverslip before being evaluated using a light microscope (Nikon) with 1,000× magnification. Two hundred spermatozoa per slide were counted. The results of functional integrity of sperm plasma membrane were expressed as the percentage of sperm with coiled tail as a result of hypo-osmotic swelling. The sperm acrosomal integrity was evaluated by staining with a fluorescein isothiocyanate peanut agglutinin: FITC-PNA utilizing a method used by Axner, Hermansson [2] with minor modifications. In brief, the semen sample (10  $\mu$ l) was placed onto a glass slide, smeared, and air-dried. The glass slide was immersed in 95% ethyl alcohol for 30 sec in order to permeabilize the sperm plasma membrane, and air-dried. The dual fluorescent solution (10  $\mu$ l) was spread over the dried smear on the glass slide. This solution was prepared by mixing 100  $\mu$ l of FITC-PNA (100  $\mu$ g/ml in phosphate-buffered saline: PBS) with 5  $\mu$ l of PI (340  $\mu$ M in PBS: Molecular Probes Inc.). This was kept at 4°C for 30 min in a dark moist chamber, and subsequently rinsed by cold distilled water. This stained slide was kept in the dark at 4°C until assessment. Two hundred spermatozoa per slide were evaluated under a fluorescence microscope (Nikon Eclipse Ni series, Nikon) with 1,000× magnification. The stained sperm was classified into two categories based on staining patterns: acrosome intact sperm and acrosome reacted sperm. The sperm presenting bright green fluorescence over the whole acrosomal area, was classified as sperm with intact acrosome. The spermatozoa showing disrupted red or green fluorescence over the acrosomal area, was classified as acrosome reacted sperm. Two hundred sperm per slide were counted, and the results were reported as the percentage of sperm with intact acrosome.

The raw semen samples, after assessment of motility, concentration and morphology, were diluted with Tris-egg yolk extender

for a final concentration of  $120 \times 10^6$  spermatozoa/ml. The diluted semen samples were packaged in 0.25 ml/ French straws (IMV Technologies) by using an automated filling and sealing machine (MPP quattro, Minitube, Tiefenbach, Germany). The semen samples were equilibrated at 4°C for 4 hr. After equilibration time, the diluted semen samples were evaluated before freezing for percentage of sperm motility, sperm viability, and functional integrity of sperm plasma membrane. The samples were cryopreserved using a computer-controlled machine (Digitcool, IMV Technologies). After the cryopreservation process, the straws were immediately plunged into liquid nitrogen (-196°C), and later stored in liquid nitrogen tank for further evaluation. For thawing, three random straws of frozen semen sample from each bull, and from each lot number of frozen semen production, were thawed in water bath at 37°C for 30 sec. The frozen-thawed samples were incubated at 37°C for evaluation of percentages of sperm motility, sperm viability, functional integrity of sperm plasma membrane, and acrosomal integrity. The statistical analyses were performed using R commander (version 3.5.2). Descriptive statistics were used to describe semen quality before and after applying FM. The semen quality before and after using FM, were compared using paired *t*-test. The differences with *P*-value <0.05 were regarded as statistically significant.

The quality of fresh, pre-frozen and frozen-thawed semen are presented in Table 1. The average semen volume, the average sperm concentration, and the average total sperm output per ejaculate of fresh semen, were significantly improved after the application of FM (*P*<0.05). Total motility of fresh, pre-frozen, and frozen-thawed semen were all significantly improved after the application of FM (*P*<0.05). Progressive motility of fresh semen collected with and without FM was not significantly different. However, progressive motility of pre-frozen and frozen-thawed semen was significantly higher when FM were used (*P*<0.05). Viability of sperms did not differ significantly between the two treatment groups in fresh and pre-frozen semen samples (*P*>0.05). However, sperm viability in frozen-thawed semen samples collected with the use of FM was significantly higher compared to that of samples collected without FM (*P*<0.05). When FM were used, the mean percentage of sperms with intact plasma membrane (using HOST) in fresh, pre-frozen, and frozen-thawed semen were 63.69, 51.25, and 57.21, respectively. In contrast, the absence of FM, on average, resulted in 54.3, 44.77, and 49.03% of sperms with intact plasma membrane, in fresh, pre-frozen, and frozen-thawed semen respectively (Table 1). The impact of using FM was found to be statistically significant in all stages of semen evaluation (*P*<0.05). Furthermore, using FM did not significantly impact the percentage of spermatozoa with intact acrosome in frozen-thawed samples.

The use of FM in sexual preparation of bulls prior to semen collection is known to improve quality parameters of fresh semen, such as semen volume, sperm concentration, as well as sperm output per ejaculate [1, 6, 9, 17]. However, previous studies have mostly focused on fresh semen, and the impact of using FM on the quality of frozen-thawed semen has not been thoroughly investigated. Before investigating whether the use of FM impacted frozen-thawed semen quality, it was important to confirm its impact on fresh semen, in order to validate how FM were used during sexual preparation. Evaluation of fresh semen confirmed that using FM during sexual preparation improved several semen quality parameters including semen volume, sperm concentration, as well as total sperm output per ejaculate. These results accord with previous studies that demonstrate improvements in sperm concentration [1, 6, 9], semen volume, and total spermatozoa output per ejaculation [1, 6, 9, 17], as a consequence of using FM

**Table 1.** Descriptive statistics demonstrating impact of using false mounts (FM) on semen quality parameters

Semen quality parameter	Without FM (n=30)			With FM (n=30)			Significance (P-value)
	Mean	SD	Range	Mean	SD	Range	
<b>Fresh semen</b>							
Volume (ml)	4.43	2.03	1.5–8.0	5.50	2.02	2.0–9.5	<0.05
Concentration ( $\times 10^6$ sperm/ml)	1,129	361.95	535–1,840	1,241	282.08	682–1,774	<0.05
Total Sperm output ( $\times 10^6$ sperm)	5,253	3,227.08	1,340–10,026	7,096	3,559.53	1,600–16,853	<0.001
Total motility (%)	74.63	10.03	49.20–89.60	80.10	6.29	70.60–92.60	<0.05
Progressive motility (%)	50.20	11.51	11.50–70.10	52.07	10.42	28.50–69.80	NS <sup>c)</sup>
Sperm viability–eosin/nigrosin (%)	79.93	11.65	42.5–95.0	79.07	7.57	63.5–91.5	NS <sup>c)</sup>
HOST (%) <sup>a)</sup>	54.3	14.76	20.0–81.5	63.69	10.13	41.5–79.5	<0.05
<b>Pre-frozen semen</b>							
Total motility (%)	68.11	15.13	31.10–90.70	75.90	11.45	43.40–92.30	<0.05
Progressive motility (%)	35.63	11.50	12.90–57.80	43.03	13.47	20.50–63.20	<0.05
Sperm viability–eosin/nigrosin (%)	79.87	7.47	55.50–91.00	81.78	4.98	69.00–88.50	NS <sup>c)</sup>
HOST (%) <sup>a)</sup>	44.77	13.00	14.00–72.50	51.25	12.47	25.50–76.00	<0.05
<b>Frozen-thawed semen</b>							
Total motility (%)	34.67	11.23	10.50–52.40	39.63	14.40	9.30–69.60	<0.05
Progressive motility (%)	11.62	4.66	3.40–24.30	13.64	5.73	3.00–24.10	<0.05
HOST (%) <sup>a)</sup>	49.03	10.24	26.00–66.50	57.21	7.93	41.50–74.00	<0.001
Sperm viability-SYBR14/PI (%)	34.87	12.00	5.50–59.50	44.47	15.91	11.00–74.50	<0.001
FITC (%) <sup>b)</sup>	72.55	12.59	47.50–89.00	71.88	18.27	32.00–96.50	NS <sup>c)</sup>

a) HOST=Functional integrity of sperm plasma membrane. b) FITC=Percentage of spermatozoa with intact acrosome. c) NS=Not significant (*P*-value >0.05).

in sexual preparation. Mechanistically, these improvements are likely attributable to an increase in release of oxytocin from the posterior pituitary, which results in increased smooth muscle contractions, and consequently, improved sperm transport in the male reproductive tract at the time of ejaculation [23]. In addition to fresh semen, quality parameters of pre-frozen and frozen-thawed semen were also assessed. The use of FM significantly improved total motility of fresh, pre-frozen and frozen-thawed semen. However, significant improvements in sperm progressive motility were only observed in pre-frozen and frozen-thawed semen. Similarly, functional integrity of sperm plasma membrane was significantly higher in pre-frozen and frozen-thawed semen collected using FM. Finally, significant improvements in sperm viability were only observed in frozen-thawed semen collected via FM, and not in fresh or pre-frozen semen.

The improvements in sperm motility and viability is likely attributable to increased seminal plasma in ejaculates obtained via FM. The bull seminal plasma is a complex fluid constituted by the combined secretions of epididymis and accessory sex glands during ejaculation. Bovine seminal plasma plays crucial roles in supplying nutrients supporting sperm physiology [33]; and several studies have reported associations between the secretions of accessory sex glands or specific seminal plasma proteins, and sperm function [3, 12, 20, 24, 25, 33]. For example, PDC-109, which is secreted from seminal vesicles, is known to increase sperm motility in bulls [28]. There are also several examples of seminal plasma proteins conferring protection to sperm plasma membranes, particularly during cryopreservation. Therefore, increased secretion of seminal plasma proteins with the use of FM may also explain significantly improved functional integrity of sperm plasma membranes observed at all stages (fresh, pre-frozen and frozen-thawed); and significantly improved sperm viability that was specifically observed in frozen-thawed semen. Increased presence of proteins that offer protection against damage associated with cryopreservation likely explains why significant improvement in sperm viability were only observed in frozen-thawed semen, and not fresh or pre-frozen semen. In this context, it is also important to note that sperm viability in frozen-thawed semen was assessed via SYBR-14/PI staining, as opposed to eosin-nigrosin staining, which was used to assess sperm viability in fresh and pre-frozen semen samples. However, given that SYBR-14/PI staining is more effective than eosin-nigrosin (PMID: 10732141), it is unlikely that the significantly higher sperm viability observed in frozen-thawed semen is an artefact. Overall, these results suggest that, apart from improving quality of fresh semen, the use of FM in sexual preparation may offer additional benefits in protecting sperms through cryopreservation.

Semen extenders are also specifically designed to counter the adverse effects of cryopreservation on semen. Cryopreservation, for example, can destabilize sperm plasma membranes by altering permeability and lipid composition [18, 31]; cause premature capacitation of the bull sperm [10]; and induce acrosomal reaction of the sperm [16]. Egg yolk, one of the main constituents of semen extenders, is rich in lipids that can protect against these adverse effects [18]. In our study, while the use of FM positively impacted the functional integrity of sperm plasma membranes, no such impact was observed on acrosomal integrity. It is possible that seminal plasma proteins play a greater role in maintaining plasma membrane integrity, and semen extenders play a greater role in maintaining acrosomal integrity. This would explain why FM only seemed to have an impact on plasma membrane integrity and not acrosomal integrity.

In terms of limitations, it is possible that variability between individual bulls contributed to the variability observed in semen quality parameters in this study. To minimize this source of variation, we used bulls of the same breed and age group that were kept under similar management and housing conditions. Similarly, the time of semen collection, variability in seminal plasma proteins contained in ejaculates from different bulls [21]; and between repeated ejaculates of the same bull [31], are other sources of variation that could influence results of this study. However, our approach of using a paired *t*-test design should account for some of these variations and afford greater confidence in our results.

Improvements observed in semen quality parameters obtained via FM provide some validation of how FM were applied during sexual preparation. However, the application of FM in Brahman beef bulls could be investigated further to identify the optimal number required for sexual preparation, so as to have maximal improvements in semen quality parameters at all stages (i.e. fresh, pre-frozen and frozen-thawed). In the present study, bulls were subjected to the recommended three FM without restraint [7]. From our observations, the bulls in this study spent a lot of time for sexual preparation by three FM. The *Bos indicus* breeds are generally considered to have lower libido than *Bos taurus* breeds, and the beef breeds may express less sexual activity than the dairy breeds [8, 26]. Moreover, the sexual behavior, such as the reaction time and the total time taken in mounts, was correlated with semen quality parameters in the bulls [30]. We recommend that the optimal number of FM during semen collection in Brahman bulls should be further investigated to compare frozen-thawed semen quality after the applications of different number of FM.

In conclusion, the results of the present study demonstrate that the application of FM prior to bull semen collection, significantly improved many of the fresh, pre-frozen, and frozen-thawed semen quality parameters. Moreover, using FM improved sperm viability of post-freeze semen, which is a novel finding, and requires further investigation. Further studies are required to identify and quantify variation between different seminal plasma proteins after application of optimal number of FM, which may in turn have an impact on the quality of frozen-thawed semen.

**ACKNOWLEDGMENTS.** The present study was funded and supported by grants of Northeastern Region Standard Semen Production Center as well as Semen Quality Control and Research Section, Bureau of Biotechnology in Livestock Production, Department of Livestock Development (DLD), Thailand. This study was also funded and supported by Office of the Civil Service Commission (OCSC), Thailand.

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