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# Relationship between sperm penetration distance in cervical mucus and frozen semen characteristics vis-à-vis buffalo bull fertility

A K SINGH[1], P S BRAR[2] and RANJNA S CHEEMA[3]

*Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab 141 004 India*

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Bovine cervical mucus is used in *in vitro* sperm penetration test for evaluating semen quality and in prediction of fertilizing capacity of spermatozoa (Anilkumar *et al.* 2001). The test is based on visual assessment of linear distance covered by the foremost sperm cell (vanguard spermatozoa) in capillary tube. For optimum fertility, a bull spermatozoa is expected to penetrate beyond 20 mm in cervical mucus from the base of capillary tube for this kind of assessment (Ivic *et al.* 2002). However, most *in vitro* studies for assessment of sperm penetration distance do not exhibit sufficient determination of bull fertility (Ola *et al.* 2003, Gillan *et al.* 2008). In addition to penetration distance, sperm count in cervical mucus is also linked to semen fertility (Tas *et al.* 2007). Furthermore, vital spermatozoal characteristics such as motility, morphology, acrosome and DNA integrity and acrosome reaction significantly affect sperm penetration and number in cervical mucus (Verberckmoes *et al.* 2002, Zodinsanga *et al.* 2015). However, mutual correlation studies between migration capacity of spermatozoa in mucus, seminal attributes and fertility are still meager in buffalo bulls. A combination of all variables could present a higher predictive value. Keeping in view above facts, the present study was undertaken to study and compare the relationship of sperm penetration distance in cervical mucus with frozen semen quality and subsequent fertility of buffalo bulls.

*Procurement of frozen buffalo bull semen:* Semen of 30 healthy breeding Murrah buffalo bulls (aged between 4–8 years), frozen from same ejaculate and on same date in straws of 0.25 ml each containing $25\times10^6$ spermatozoa were collected and earmarked for the present study. The study was conducted in September having ambient temperature 30.6°C and relative humidity 92%. All bulls were maintained at 2 Government bull farms in Punjab (Semen Bank, Nabha and Semen Bank, Khanna) and housed under same raising and feeding conditions. Semen was collected twice a week through artificial vagina method. None of the bull selected for this study had any preceding physical abnormalities.

Present address: [1]Assistant Professor (assengar2001@yahoo.co.in), Department of Veterinary Gynaecology and Obstetrics, [2]Professor-cum-Head (parkashbrar@gmail.com), [3]Senior Physiologist-Reproduction (ranjna.cheema@gmail.com).

*Collection and evaluation of mucus:* Cervical mucus was collected from healthy, cyclic estrus buffalo under aseptic conditions and selected on the basis of elasticity (spinbarkeit) and degree of crystallization. White side test using 5% sodium hydroxide was performed to evaluate the mucus sample for infection, if any. Only clear and fresh mucus showing typical fern pattern was used for conducting experiment.

*Preparation of test system:* A loading manifold from a scalp vein set and a 5 ml disposable syringe was prepared for filling non-heparinized hematocrit capillary tubes with cervical mucus. The needle from one end of scalp vein set was replaced by a capillary tube and other end was attached to syringe. The cervical mucus was emptied into a petri dish and aspirated into capillary tube by syringe. The trailing mucus was cut with scissor leaving a small amount protruding from filled end of tube. Capillary tube was removed from loading manifold and plugged from one side using polyvinyl alcohol (PVA) powder. The tube was allowed to stand for 10 min at 37°C. Thereafter, 5 straws/bull were emptied in an eppendorf tube (2 ml). The open end of capillary tube was placed 5 mm below the surface of semen in the eppendorf tube and incubated at 37°C for 1 h. After incubation, the tube was removed from semen, fixed on a previously calibrated glass slide and viewed under microscope (400×). The length of tube was scanned to establish farthest distance from semen reservoir travelled by spermatozoa. The maximum penetration distance travelled in millimeters (mm) by the most progressive or vanguard spermatozoa after 1 h of incubation was defined as sperm penetration distance. The numbers of penetrated spermatozoa were counted in the peak segment of 0.5 cm.

*Classification of sperm penetration distance:* The cervical mucus penetration test was performed and graded as per Hollinshead *et al.* (2003). Based on penetration distance, semen samples from all bulls were divided into 2 groups, viz. greater penetration distance (GPD, > 20 mm; n, 15) and lesser penetration distance (LPD, < 20 mm; n, 15).

*Evaluation of semen parameters:* The acrosome integrity and acrosome reaction of the frozen-thawed spermatozoa were evaluated by staining air-dried smears with Giemsa stain, using a modification of the basic TALP medium for acrosome reaction (Yanagimachi 1994). Capacitation status was assessed at every 2 h interval for 6 h. The functional integrity of spermatozoa was determined by hypo-osmotic swelling test using hypo-osmotic solution (100 mOsm/l), viability and morphology through Eosin-Nigrosin staining technique, DNA integrity with Acridine Orange (Lui and Baker 1992) and motility and kinetic parameters through a previously validated computer assisted semen analysis (CASA; version Hamilton-Thorne IVOS 12.2). All functional tests were replicated three times per bull semen. At least 200 spermatozoa were counted in each replicate for different pattern of tests. The number of spermatozoa was converted to percentage. The mean of 25 scans for CASA parameters and 3 replicates/bull semen for per cent viability, abnormality, HOST, DNA integrity, acrosome reaction, penetration distance and sperm count was used for statistical analysis.

*Bull fertility:* Buffaloes (300) were synchronized using double ovsynch protocol (PGF2β-GnRH-PGF2α-GnRH on day –2, 0, 7 and 9, respectively) followed by fixed time inseminations at 16 and 40 h after last GnRH injection. Fertility was expressed as first service conception rate (FSCR) and determined as the ratio of number of buffaloes that conceived after first insemination to total number of first services of buffaloes.

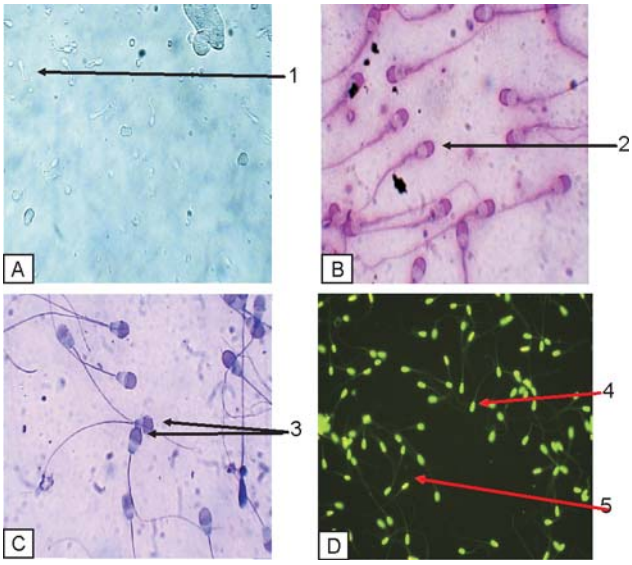


Fig. 1 **(A-D)**. Frozen-thawed semen characteristics of buffalo bulls (**A**, HOST; **B**, Acrosome integrity; **C**, Acrosome reaction; **D**, DNA integrity). **A**, Arrow 1, Hos -ve spermatozoa with curled tail; **B**, Arrow 2, Giemsa stained spermatozoa with intact acrosome; **C**, Arrow 3, Giemsa stained spermatozoa with swelling and vesiculation; **D**, Arrow 4, acridine orange stained spermatozoa with green fluorescence on nucleus showing intact DNA; Arrow 5, acridine orange stained spermatozoa with orange fluorescence on nucleus showing damaged DNA.

*Statistical analysis:* The statistical analysis was performed with Statistical Package for Social Sciences (SPSS, version 16.0) program. The proportionality data were transformed using the arcsine transformation with adjustment to allow for zero values. The mean ± SE were calculated using arcsine transformed data in the software. The independent samples t-test was used for comparing the level of significance of different parameters between 2 penetration distance groups (GPD and LPD). Coefficients of correlation were analyzed by Pearson correlation coefficients between sperm penetration and semen characteristics for both groups. The minimum significant interaction was considered at 5% level.

*Assessment and comparison of semen characteristics and buffalo bull fertility in sperm penetration distances:* The frozen-thawed semen from all bulls was assessed for semen characteristics, motion and kinetic traits (objectively analyzed through CASA) and bull fertility (FSCR) in greater and lesser penetration groups (Table 1). Spermatozoa from bulls with GPD displayed significantly ($P<0.05$) higher percentage of motile spermatozoa (total and progressive), acrosome integrity, acrosome reaction, FSCR (bulls) and an ability to penetrate cervical mucus in more numbers as compared to their counterparts (Fig. 1). Similar studies (Phillips *et al.* 2004, Zodinsanga *et al.* 2015) in cattle bulls reported that penetration of spermatozoa in cervical mucus depends upon sperm motility, viability, acrosome and

Table 1. Comparison of frozen-thawed spermatozoal characteristics and fertility of buffalo bulls in greater and lesser penetration distance

| Parameter \ Penetration distance | GPD (≥ 20 mm, n = 15) | LPD (< 20 mm, n = 15) |
|---|---|---|
| Total motility (%) | 49.4 ± 2.3$^a$ | 41.5 ± 1.9$^b$ |
| Progressive motility (%) | 39.9 ± 1.6$^a$ | 26.2 ± 1.5$^b$ |
| Viability (%) | 71.1 ± 2.4 | 67.5 ± 2.3 |
| Abnormality (%) | 15.2 ± 1.9 | 14.6 ± 1.9 |
| HOST (%) | 66.7 ± 1.7 | 67.0 ± 2.1 |
| Acrosome integrity (%) | 75.0 ± 1.4$^a$ | 71.0 ± 1.6$^b$ |
| DNA integrity (%) | 77.3 ± 2.3 | 80.0 ± 3.2 |
| Acrosome reaction (%) | 54.5 ± 2.6$^a$ | 48.9 ± 2.5$^b$ |
| FSCR (%) | 44.0 ± 4.2$^a$ | 30.0 ± 4.1$^b$ |
| Sperm number in 0.5 cm peak segment | 182.2 ± 7.0$^a$ | 164.5 ± 7.6$^b$ |
| Average path velocity (μm/s) | 106.2 ± 4.6 | 101.2 ± 4.0 |
| Straight line velocity (μm/s) | 86.8± 3.4 | 84.2 ± 3.2 |
| Curvilinear velocity (μm/s) | 169.7 ± 6.4 | 166.6 ± 5.1 |
| Amplitude of lateral head displacement (μm) | 6.3 ± 0.5 | 6.9 ± 0.6 |
| Beat cross frequency (Hz) | 35.0 ± 0.7 | 34.8 ± 0.6 |
| Straightness (%) | 82.3 ± 1.1 | 83.4 ± 1.0 |
| Linearity (%) | 54.1 ± 1.7 | 53.6 ± 1.7 |

Values in the same row with different superscripts (a and b) differ significantly ($P < 0.05$). VAP, average path velocity; VSL, straight line velocity; VCL, curvilinear velocity; ALH, amplitude of lateral head displacement; BCF, beat cross frequency; STR, straightness; LIN, linearity.

Table 2. Correlation coefficients (test of significance) of two sperm penetration distances with seminal attributes, kinematics and fertility parameters of buffalo bulls

| Parameter | GPD | | LPD | |
|---|---|---|---|---|
| | R | P | R | P |
| Total motility | 0.396[a] | 0.031 | 0.352[b] | 0.048 |
| Progressive motility | 0.629[a] | 0.006 | 0.563[b] | 0.018 |
| Viability | 0.462 | 0.061 | 0.387 | 0.072 |
| Abnormality | -0.616 | 0.035 | -0.632 | 0.042 |
| HOST | -0.529[a] | 0.029 | -0.463[b] | 0.037 |
| DNA integrity | 0.379 | 0.171 | 0.298 | 0.208 |
| Acrosome integrity | 0.768[a] | 0.002 | 0.549[b] | 0.008 |
| Acrosome reaction | 0.416[a] | 0.044 | 0.392[b] | 0.038 |
| FSCR | 0.523 | 0.005 | 0.448 | 0.007 |
| Sperm number | 0.271[a] | 0.019 | 0.132[b] | 0.026 |
| Average path velocity (μm/s) | 0.432 | 0.052 | 0.386 | 0.060 |
| Straight line velocity (μm/s) | 0.527[a] | 0.023 | 0.491[b] | 0.068 |
| Curvilinear velocity (μm/s) | 0.383 | 0.137 | 0.267 | 0.319 |
| Amplitude of lateral head displacement (μm) | -0.406 | 0.302 | -0.421 | 0.205 |
| Beat cross frequency (Hz) | 0.538 | 0.076 | 0.376 | 0.247 |
| Straightness (%) | 0.613 | 0.071 | 0.519 | 0.064 |
| Linearity (%) | 0.331 | 0.062 | 0.503 | 0.083 |

Values in the same row with different superscripts (a and b) differ significantly ($P < 0.05$). VAP, average path velocity; VSL, straight line velocity; VCL, curvilinear velocity; ALH, amplitude of lateral head displacement; BCF, beat cross frequency; STR, straightness; LIN, linearity.

membrane integrity and capacitation status. Further, spermatozoa with higher proportion of quality semen attributes have better chances to reach the site of fertilization and fertilize the oocyte (Muino-Blanco *et al.* 2008). Given this fact, the ability of spermatozoa to migrate through female reproductive tract was mimicked using migration in cervical mucus in the present study. Although non-significant, a higher (P>0.05) percentage of viable spermatozoa and vital sperm kinetic parameters, viz. average path velocity (VAP), straight line velocity (VSL) and curvilinear velocity (VCL) along with lower percentage of straightness (STR) were observed in GPD group than in their contemporary mates. Spermatozoa with high VAP and VSL but lower STR had greater ability to migrate through cervical mucus and demonstrated high motility and intact acrosome (Martinez-Rodriguez *et al.* 2012). Therefore, progression of more actively motile spermatozoa with intact acrosome, better capacitation status and sperm kinematic parameters through cervical mucus in GPD than those with LPD might be responsible for higher fertility of buffalo bulls in the former.

*Correlation of* in vitro *sperm progression distances with semen quality and bull fertility:* The correlation coefficients of 2 sperm penetration groups in cervical mucus with corresponding sperm characteristics, kinetic and FSCR are presented in Table 2. The percentage of total and progressive motility, acrosome integrity and acrosome-reacted spermatozoa were significantly correlated with both penetration distances although, the association was more noticeable in GPD than in LPD group. The results are in consonance to the findings of Anilkumar *et al.* (2001) who demonstrated that acrosome integrity (r, 0.94; P<0.01) and motility (r, 0.88; P<0.01) were significantly and positively correlated with sperm penetration in mucus and predicted about 38% and 54% of variation in distance penetrated, respectively. Likewise, a significant positive correlation existed for sperm number and bull fertility in greater and lesser penetration distances (r, 0.271; P, 0.019; r, 0.132; P, 0.026 and r, 0.523; P, 0.005; r, 0.448; P, 0.007; respectively). Similar studies (Ulcova-Gallova 2010) in cattle bulls also showed a significant positive correlation of penetration distance (r, 0.47; P<0.05) with number of spermatozoa that migrated in cervical mucus (r, 0.59; P<0.01) and non-return rates of bulls (r, 0.24; P<0.05). On the other hand, a significant negative relationship was found with percentage of abnormal spermatozoa and damaged membrane integrity (HOST) in GPD group (r, –0.616; P, 0.035; r, –0.529; P, 0.029) and LPD (r, –0.632; P, 0.042; r, –0.463; P, 0.037). Likewise, no significant correlations of percent viability and DNA integrity were observed with two sperm penetration distances. These findings are also in agreement with the observations of Martinez-Pastor *et al.* (2008) who observed loss of viable spermatozoa (r, 0.16; P, 0.132), morphologically normal spermatozoa (r, 0.37; P, 0.416) and DNA activity (r, 0.22; P, 0.381) were not directly related to sperm progression in mucus. Within kinetic parameters, VSL showed a significant positive correlation with spermatozoa of GPD (r, 0.527; P, 0.023) as compared to those with LPD (r, 0.491; P, 0.068) in cervical mucus. In general, most correlations were moderate, positive and higher in GPD than in their counterparts. Similar observations in dairy bulls were recorded by Gillan *et al.* (2008) who demonstrated the importance of VSL in the success of cervical mucus penetration and its association with fertility. They further speculated that a high VSL might be important in sperm transport through female reproductive tract and penetration of oocyte vestments. Eventually, a positive relationship of *in vitro* sperm progression distance with semen quality and bull fertility may form a cause of improved reproductive performance in GPD group.

## SUMMARY

In this study, relationship of sperm penetration distance in mucus was assessed and compared with spermatozoal characteristics, utilizing frozen semen samples obtained from 30 buffalo bulls. Quality semen parameters (motility, kinematics, morphology, viability, HOST, acrosomal and DNA integrity, acrosome reaction) in conjunction with first service conception rate (FSCR) of bulls were determined. Based on penetration distance in buffalo mucus, semen

samples were divided into 2 groups, viz. greater penetration distance (GPD, > 20 mm; n, 15) and lesser penetration distance (LPD, < 20 mm; n, 15). Most spermatozoal characteristics were significantly (P<0.05) higher in GPD than in LPD group. The spermatozoa of GPD and LPD were significantly and positively correlated with total motility, progressive motility, acrosome integrity and acrosome reaction. Alternatively, a negative relationship was found with percentage of abnormal spermatozoa and damaged membrane integrity (HOST) in the two penetration distances. In conclusion, significant positive correlations occurred between sperm penetration distance, semen characteristics and fertility parameters. A combination of factors must, therefore, be used to predict the bull fertility.

In conclusion, significant positive correlations existed between sperm penetration distance, quality semen characteristics and fertility parameters. The distance penetrated by spermatozoa capable of going beyond 20 mm in an optimized sperm migration test, could be a useful parameter for *in vitro* evaluation of sperm quality and subsequent fertility of buffalo bulls.

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