Influence of the Different Batches of Estrous Cervical Mucus Mixed for Homogenization on Penetration by Spermatozoa in Cattle

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Abstract. This study examined differences in the penetration of various batches of mixed cervical mucus by bull spermatozoa. Mucus was collected from different numbers (2, 2, 3, 4 or 7) of cows or heifers in estrus and a part of the sample was individually, and the rest was mixed, and stored frozen. The distance traveled by the most advanced sperm cell in frozen-thawed mucus during incubation at 38°C for 10 min was measured (sperm penetration). Variation of sperm penetration in the individual mucus samples was reduced by mixing but the difference among the batches of mixed mucus was significant (p<0.0002). Nevertheless, the number of animals from which mucus was collected and mixed did not seem to influence the sperm penetration of the different batches. Unexpectedly it was observed that some individual and mixed mucus samples had reduced viscosity after freezing and thawing and sperm penetration in the mixed mucus samples showed a significant, negative correlation with the proportion of mucus of reduced viscosity contained in the batch (r=–0.969, p<0.01). These results suggest that the difference in the sperm penetration of mixed mucus may probably be rather independent of the number of mucus donors.

Key words: Cervical mucus, Bull, Spermatozoa, Penetration, Semen

Cattle reproduction is largely dependent upon artificial insemination, and low fertility of bulls proved after insemination may be one of the most important problems. To predict or diagnose bull subfertility or infertility, analysis of semen is essential and also is an important step in the preparation of frozen semen. Among the standard analyses, sperm motility may be one of the most valuable parameters but this is usually examined subjectively by a skilled technician, and is not always an accurate estimation of bull fertility after artificial insemination.

The mucus penetration test, in which penetration of cervical mucus by spermatozoa is quantified, has been employed in the diagnosis of human male infertility [1, 2] and the availability of this test to examine Holstein bull subfertility has also been suggested [3] but variation in the distance traveled by spermatozoa among the samples of mucus collected from various donors has been pointed out [4, 5] and this makes evaluation of spermatozoa from different bulls difficult in this test. A previous study has suggested that mucus collected from various donors and mixed for homogenization can be employed for the mucus penetration test with reasonably low intra- and inter-assay coefficients of
variation [6] but the difference in the penetration distance in batches of mixed mucus was not clear. This study therefore examined the effect of mixing mucus collected from a number of donors on the penetration of mucus by bull spermatozoa.

**Materials and Methods**

**Semen**

An ejaculate was collected from a mature Japanese beef bull of proven fertility (12 years old at the time of semen collection) with an artificial vagina, diluted, loaded into 0.5-ml straws and stored frozen in liquid nitrogen by a standard method. A straw was allowed to thaw at 39°C for a few minutes and an aliquot (0.1 ml) was used for the mucus penetration test. For all the experiments carried out in this study, straws of the one ejaculate were used.

**Cervical mucus**

Cervical mucus was collected from a total of 38 Holstein cows or heifers in standing estrus by aspirating from the vagina with a plastic catheter attached to a 50-ml syringe and transported to a laboratory at about 5°C. The mucus was examined for volume, viscosity and appearance [3] and colorless, transparent samples or those with a small limited cloudy part and good viscosity were further examined for the presence of foreign cells by observing the mucus in rectangular capillary tube under a microscope at 100×. Colored or entirely cloudy samples were excluded. Samples collected from 21 cows or heifers (55%) passed the examination, and 15 were used in this study. A part of each mucus sample was individually aliquoted into small portions (0.5 ml each), frozen and stored in liquid nitrogen by the previously reported method [5], and the rest was combined and mixed by gently cutting several times with surgical scissors in a glass beaker [3]. The mixed mucus was then aliquoted, frozen and stored in liquid nitrogen as described above. A total of 5 batches of mixed cervical mucus were stored and had been prepared from mucus samples from 2, 2, 3, 4 or 7 animals.

**Mucus penetration test**

Mucus penetration test was carried out by the upright 38°C method as described previously [7]. Briefly, a small volume of H2O and a plastic vial with the lid cut off were consecutively placed in a conical test tube. A vial of frozen mucus was allowed to thaw at room temperature for 20–30 minutes and loaded into a rectangular glass capillary tube (inside diameter, 0.4 × 4 mm; length, 100 mm; Vitro Dynamics, Rockaway, USA). Either tip of the tube was closed with clay (Hematoseal, Sherwood, Japan) and placed in the vial contained in the test tube. Thereafter, a portion of frozen-thawed semen was added to the vial to bring it into contact with the surface of the mucus and was covered with warm paraffin oil. After 10 min of incubation at 38°C, the capillary tube was immediately transported to another water bath at 60°C and kept for 5 min to immobilize the spermatozoa. The tube was put onto a calibrated glass slide and spermatozoa that penetrated the mucus were examined under a light microscope (100×). The sperm cell that had advanced furthest in the tube was found and the distance was measured (mm/10 min). The assay was carried out in duplicate and sperm penetration was expressed as the average for the two measurements.

**Statistical analyses**

Results are expressed as the average ± standard error of the mean (SEM). Data were analyzed by one-factor ANOVA, Student’s t-test or simple regression analysis and the difference with p<0.05 was regarded as statistically significant.

**Results**

Table 1 shows the results of the mucus penetration test. As seen with the SEM, penetration in individual mucus samples varied widely but that of mixed mucus showed less variation within the batch except on one occasion (The top row of the table). Nevertheless, mucus penetration was different significantly among the mixed mucus batches (p<0.0002, 1-factor ANOVA). A tendency was noticed for sperm penetration of mixed mucus samples to depend on those in individual samples that constituted the mixed ones. Penetration of mixed mucus tended to be slightly greater than that of individual mucus samples. No clear relation was seen between the number of donors from which mucus was combined and the penetration of the mixed mucus batches.
Unexpectedly it was observed that some of the individual samples or one of the mixed samples (Batch No. 1 in Table 1) had greatly reduced viscosity and became water-like after freezing and thawing. This change was confirmed by observing that after incubation, many spermatozoa were found in the tube with heads directed at random, and it was considered that the spermatozoa had not penetrated the mucus but semen itself had slipped into the mucus or mucus-capillary tube interface passively. By contrast, when viscosity was not apparently changed after freezing and thawing, only one top sperm cell was found among the spermatozoa penetrating the mucus, i.e. the increasing number of spermatozoa followed the top cell in the mucus. With the increasing content of individual mucus samples showing reduced viscosity in the total volume of the mixed samples, sperm penetration was decreased (Table 1).

Discussion

Because of differences in the properties of individual mucus samples, the use of a synthetic medium as substitute has been explored, but penetration by spermatozoa was less than in cervical mucus [8, 9]. Therefore, although a synthetic substitute is standardized and more available, cervical mucus would be more suitable in comparing penetration by sperm from various bulls.

When cervical mucus is collected and stored for the test, large amounts of mucus of standardized quality should be kept for use at all times. Although a useful kit for the mucus penetration test, where bovine cervical mucus is standardized, has been successfully introduced for the assessment of human sperm function [10], attempts in cattle have not succeeded in correlating the penetration distance with the conception rates [11–13]. It is therefore considered that assessment of bull spermatozoa by mucus penetration test may not be as easy as in human spermatozoa and it is very important that the difference in the penetration distance among different bulls be maximized in order to distinguish fertile from sub- or infertile spermatozoa. Kummerfeld et al. [4] reported that sperm migration was more unidirectional and longer in the mucus stored in a beaker and loaded into capillary tubes shortly before penetration started (bulk storage) than mucus frozen and thawed in the same capillary tube used for sperm penetration (capillary tube storage), and this suggested that mucus should be loaded into capillary tubes just before the sperm penetration assay is performed. In fact, the penetration distance of bull spermatozoa was far shorter in the report by Galli et al. [13] (Average 28.5 mm/90 min at 37 °C), who used a commercial kit employing capillary tube storage, than the results obtained in this study (Table 1) and others [3, 7]. In this respect, the bulk storage method may be superior to achieve maximal sperm penetration and this was the reason why the present study attempted to establish the best freezing method for bulk storage of large amounts of cervical mucus in vials.

Another problem may be variation in sperm penetration distance in the different batches of mixed mucus (Table 1). It was expected that different batches of mixed mucus varied because of differences among mucus donors [4, 5]. The only example in which SEM in the mixed mucus was relatively high might be due to a high proportion (100%) of mucus with reduced viscosity in the mixed mucus batch (Table 1). The observation that

<table>
<thead>
<tr>
<th>Batch No.</th>
<th>No. of mucus samples mixed</th>
<th>Ratio of mixture</th>
<th>No. of samples with reduced viscosity</th>
<th>Amount (%) of mucus showing reduced viscosity to total volume</th>
<th>Penetration of individual mucus sample (mm/10 min)</th>
<th>Penetration of mixed mucus sample&lt;sup&gt;1&lt;/sup&gt; (mm/10 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>23 : 7</td>
<td>2</td>
<td>100</td>
<td>31.7 ± 2.3</td>
<td>36.1 ± 3.1</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>30 : 23</td>
<td>0</td>
<td>0</td>
<td>48.9 ± 4.5</td>
<td>55.2 ± 0.8&lt;sup&gt;ad&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>1 : 1 : 1</td>
<td>0</td>
<td>0</td>
<td>49.7 ± 3.5</td>
<td>52.8 ± 1.1</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>10 : 10 : 7 : 3</td>
<td>2</td>
<td>43</td>
<td>44.8 ± 3.0</td>
<td>45.9 ± 0.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
<td>7</td>
<td>1 : 1 : 1 : 1 : 1 : 1 : 1</td>
<td>2</td>
<td>29</td>
<td>48.2 ± 3.2</td>
<td>52.5 ± 0.9&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup> The correlation between content and penetration was significant (r=–0.969, p<0.01).
<sup>2</sup> Number of replicates was 3 and difference among batches is significant (p<0.0002, one-factor ANOVA).
<sup>ad</sup> Different superscripts indicate significant differences (p<0.04, t-test).
the SEM in the individual mucus samples was reduced by mixing re-confirmed that the mixing method employed in the previous [3, 6] and this study is not unsuitable. Rather, the difference may have been due to the inclusion of mucus samples with reduced viscosity of the mixture. No previous report has described this change and the reason for this is as yet unknown. It would be possible that some mucus had low tolerance for the freezing and thawing processes. Although mucus was carefully selected for suitability prior to freezing, because some of the mucus samples had reduced viscosity, the sperm penetration distance in individual or mixed mucus was decreased. This would have caused the variation in sperm penetration among different batches of mixed mucus. A finding showing that the difference among the mixed batches was correlated with the amount of mucus of reduced viscosity in them rather than the number of donors from which mucus was collected suggests that, if only mucus samples with viscosity unchanged after freezing and thawing were mixed and stored, the difference among the batches might be greatly reduced. Not many donors would then be required to obtain large amounts of mucus with similar properties and the number of donors would not be very important. A possible way to mix mucus samples of unchanged viscosity would be that when mucus is collected, each sample is first frozen-thawed and examined for viscosity and the samples known to have good viscosity after freezing and thawing may be mixed, aliquoted and stored for use in the assay. Further experiments will be required to test this possibility and to correlate bull fertility with sperm penetration into bulk stored cervical mucus.

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