CONTRIBUTION OF SEMINAL PLASMA, SPERM NUMBERS, AND GAS PHASE TO DILUTION EFFECTS OF BOVINE SPERMATOZOA

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ABSTRACT

Semen stored at 200 million sperm per milliliter at 5 C survived storage significantly better (P < 0.01) than semen stored at 12.5 million. Both additional seminal plasma and dead sperm significantly depressed livability (P < 0.01) in highly diluted samples. However, this difference between dilution rate of storage was eliminated when diluents were saturated with nitrogen, and a decline in storage life, similar to that exhibited by highly diluted sperm, could be simulated in sperm stored at 300 million per milliliter in a diluent gassed with oxygen. It is concluded that the dilution effects observed are due to availability of O2 to sperm, and that the protective action of nitrogen saturation lies in the reduction of O2 tension in the diluting media. Field trials showed an advantage for storage at 200, but rediluted to 12.5 million sperm per milliliter immediately before use, compared to storage at 12.5 million sperm per milliliter for semen used on the day after collection (difference 3.1% P < 0.025) in an unsaturated diluent, but an advantage of 11.7% (P < 0.01) in favor of storage at 12.5 million sperm per milliliter with semen used seven days after collection in a nitrogen-saturated diluent.

That highly diluted sperm do not survive as well as sperm stored in a more concentrated form is well recognized (2).

This paper reports on the contribution of sperm numbers, seminal plasma, and gas phase to dilution effects on bovine spermatozoa in diluents used in commercial artificial breeding.

MATERIAL AND METHODS

Buffers used in these investigations were 14G (4) 14GC, (3) 14GN and 14GCN (Caprogen). The composition of the buffers were 14G: 2.0% sodium citrate, 1.0% glycine, 1.25% glycerol, and 0.3% glucose, dissolved in glass-distilled water, and 14GC consisted of 14G plus 0.03125% caproic acid. The diluents were prepared by adding 20% egg yolk. The fully prepared diluents contained 1,000 IU penicillin and 1,000 μg of streptomycin per milliliter. 14GN and caprogen were prepared by bubbling nitrogen through the fully prepared 14G plus egg yolk, and 14GC plus egg yolk diluents, respectively, for 20 min at 5 C.

All samples of diluted semen were stored at 5 C. Because of difficulties in estimating the percentage of motile sperm after different periods of storage at 5 C, it was decided to measure livability by duration of life in hours at 37 C after different periods of storage. The end point of livability at 37 C was defined as when less than 10% of the sperm showed progressive forward motility. In practice this point was relatively easy to determine, for at this stage not only are there few motile sperm but the velocity of motion is much reduced.

Although length of life at 37 C is much easier to determine than percentage of motile sperm, certain difficulties are encountered in interpretation of results.

It is possible that the livability of freshly diluted sperm at 37 C may not necessarily be closely related to their ability to maintain livability at 5 C. This, however, would be reflected in a reduction of incubation life (37 C) after different periods of storage. However, livability of semen at 37 C after different periods of storage at 5 C, divided by the livability of freshly ejaculated semen at 37 C, is an index of the efficiency of the diluent to maintain livability at 5 C. If the ratio is equal to one, then length of previous storage had no effect on livability at 37 C.

This index, then, is an estimate of the rate of decline in incubation life due to storage for different periods at 5 C, and differences between diluents in this index reflect differences in rate of decline of incubation life.

Analyses have been made both of hours of livability at 37 C and of the indices. However, the indices have been shown only where these would modify, or amplify, conclusions derived from an analysis of hours of livability.
Results were analyzed using the methods outlined by Snedecor (6).

Semen was collected in all laboratory trials from randomly selected bulls currently in use at the Newstead Artificial Breeding Center.

Fertility in field trials was measured as the percentage of cows reported in calf 49 days after first inseminations.

RESULTS AND DISCUSSION

In a preparatory series of trials, split ejaculates from 20 bulls were diluted in 14G to contain either 12.5 million or 200 million total sperm per milliliter. On the day after collection (Day 2), and six days after collection (Day 7), samples of each dilution rate were incubated at 37°C. Samples taken from material stored at 200 million were rediluted to contain 12.5 million total sperm per milliliter immediately before incubation. Because both samples were incubated at the same concentration, incubation life is an accurate estimate of the effect of storage at these two concentrations.

Livability in hours at 37°C for material stored at 200 million sperm per milliliter at 5°C (incubated at 12.5 million sperm) on Day 2 was 45.8 hr and on Day 7 was 37.0 hr. Livability in hours at 37°C for material stored at 12.5 million sperm per milliliter at 5°C on Day 2 was 38.3 hr and on Day 7 was 17.8 hr. There was a highly significant difference of 13.4 hr (P < 0.01) between storage concentration in length of life at 37°C. There was also a highly significant interaction (P < 0.01) between days of storage and dilution rate on livability at 37°C. Livability at 37°C for semen stored at 12.5 million declined more rapidly than semen stored at 200 million. The index of livability (Incubation life on Day 2) for semen stored at 12.5 million or 6.25 million total sperm per milliliter significantly reduced incubated life compared to semen stored at either 12.5 million or 6.25 million total sperm per milliliter. Six and one-quarter million dead sperm per milliliter were added to one of the samples containing 6.25 million total sperm for each bull. The dead sperm were obtained from pooled samples of semen obtained from five bulls. The sperm were killed by cooling the semen to −70°C in dry ice. Because the sperm were not centrifuged, the addition of dead sperm also increased the percentage of seminal plasma in these samples. Diluents used in this experiment were 14G and 14GC.

Semen from eight bulls was diluted in 14G and eight in 14GC. The addition of 6.25 million dead sperm per milliliter significantly reduced incubated life (P < 0.01), compared to semen stored at either 12.5 million or 6.25 million total sperm per milliliter. The index of livability was also significantly lower than either of the two other treatments, indicating that the rate of decline of incubated life was hastened by addition of dead sperm. Leakage of intracellular materials from these sperm, therefore, appeared to exert a definite detrimental effect on livability, and did not reduce the dilution effect.

As neither the addition of extra seminal plasma or dead sperm reduced the dilution effect, it is concluded that the detrimental effect of dilution on livability was due to dilution of live sperm and must be due to some function of metabolism.

One possibility is that when sperm are stored
at high concentrations they may reduce oxygen tension in the surrounding media. To test this, split ejaculates from 20 bulls were stored at 5 C at concentrations of 12.5 and 200 million sperm per milliliter in 14G, 14GC, 14GN (14G saturated with nitrogen), and Caprogen (14GC saturated with nitrogen). At Days 2, 6, and 8, samples were incubated at 37 C. Samples stored at 200 million sperm per milliliter at 5 C were rediluted in the appropriate diluent to contain 12.5 million sperm per milliliter, immediately before incubation.

Analyses of the effect of caproic acid addition showed that although it significantly increased incubation life it had no significant influence on dilution rate effects. Nitrogen saturation, however, markedly reduced dilution effects. This effect of nitrogen saturation on livability is shown in Table 2. Results obtained from storage in 14G and 14GC have been pooled, and results from 14GN and Caprogen also.

The pooled average of incubated life for semen stored at 12.5 million sperm per milliliter at 5 C was 39.8, and for semen stored at 200 million sperm per milliliter, 46.0 hr. The difference of 6.2 hr was significant (P < 0.01). However, the differences was much greater for unsaturated diluents (9.9 hr) than for saturated diluents (2.6 hr). This interaction between nitrogen saturation and dilution effect was significant (P < 0.01). Moreover, the difference in incubated life between storage at 12.5 million and 200 million increased with length of storage for unsaturated diluents (5.1 hr on Day 2, 10.2 hr on Day 6, and 14.3 hr on Day 8), but did not increase with saturated diluents. The nitrogen saturation X days of storage X dilution rate interaction was significant (P < 0.05).

The effect of nitrogen saturation, therefore, was to halt the decline in incubation life associated with aging semen, and this effect was much more marked in semen stored in a highly diluted form than in a concentrated form. It seems unlikely that this effect will be due to nitrogen as such, but rather to a reduction of oxygen tension in the media.

If the reason for the decline in incubated life is due to a high oxygen tension, then it might be expected that semen stored in a concentrated form but in which the diluent was gased with oxygen would exhibit a similar decline in incubation life as highly diluted sperm. To test this, split ejaculates from ten bulls were diluted to contain 12.5 million, 300 million sperm per milliliter in 14GC, and 300 million sperm per milliliter in 14GC, through which oxygen had been bubbled for 20 min. On Day 3 oxygen was again bubbled through this sample for 5 min.

Oxygenation of the diluent reduced livability at 37 C of semen stored at 300 million per

<table>
<thead>
<tr>
<th>Days of storage at 5 C</th>
<th>Unsaturated diluents (14G and 14GC)</th>
<th>Nitrogen saturated diluents (14GN and Caprogen)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Storage conc per milliliter</td>
<td>Storage conc per milliliter</td>
</tr>
<tr>
<td></td>
<td>(hr)</td>
<td>(hr)</td>
</tr>
<tr>
<td>2</td>
<td>39.9</td>
<td>47.6</td>
</tr>
<tr>
<td></td>
<td>45.0</td>
<td>51.2</td>
</tr>
<tr>
<td>6</td>
<td>31.9</td>
<td>48.3</td>
</tr>
<tr>
<td></td>
<td>42.1</td>
<td>50.0</td>
</tr>
<tr>
<td>8</td>
<td>25.0</td>
<td>47.8</td>
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<tr>
<td></td>
<td>37.3</td>
<td>50.3</td>
</tr>
<tr>
<td>Avg</td>
<td>31.6</td>
<td>47.9</td>
</tr>
<tr>
<td></td>
<td>41.5</td>
<td>50.5</td>
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</tbody>
</table>
In incubated life in hours of samples stored at 12.5 million sperm per milliliter, 300 million sperm per milliliter, and 300 million sperm per milliliter in an oxygenated diluent at 5°C. (All samples incubated at 12.5 million sperm per milliliter)

<table>
<thead>
<tr>
<th>Days of storage at 5°C</th>
<th>Sperm concentration (millions per milliliter) during storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>12.5  300  300 + O₂</td>
</tr>
<tr>
<td>7</td>
<td>38.0  45.5  40.8</td>
</tr>
<tr>
<td>7</td>
<td>20.0  39.0  13.7</td>
</tr>
<tr>
<td>Avg</td>
<td>29.0  42.3  27.3</td>
</tr>
</tbody>
</table>

milliliter. The difference of 15 hr between oxygenated and untreated semen was highly significant (P < 0.01). The interaction between days of storage and effect of oxygen was also highly significant (P < 0.01). The difference in incubated life on Day 2 was 4.7 hr and on Day 7, 25.3 hr. This increased rate of decline of sperm in an oxygenated diluent is similar to that shown by semen stored at 12.5 million sperm per milliliter. The difference on Day 2 between sperm stored at 12.5 million per milliliter and untreated sperm stored at 300 million per milliliter was 7.5 hr and on Day 7, 19 hr.

Results in this experiment indicate that the greater decline in incubated life of sperm stored in highly diluted form could be due to the greater availability of oxygen to sperm. The detrimental effect of oxygen may be due to the formation of H₂O₂ (7) in the egg yolk diluents or to the stimulation of oxidative pathways of metabolism.

To further test the effect of storage in concentrated or dilute form in nitrogen-saturated diluents, split ejaculates from 20 bulls were diluted in Caprogen to contain either 12.5 million or 200 million sperm per milliliter. Samples were incubated (at 12.5 million sperm per milliliter) on Days 2, 7, and 14. The incubated life at Day 2 for semen stored at 12.5 million was 52.3 hr, on Day 7, 52.1 hr, and on Day 14, 39.3 hr. For semen stored at 200 million sperm per milliliter, the livabilities were Day 2, 53.3 hr, Day 7, 50 hr, and Day 14, 36.1 hr. The difference between the two methods of storage was not significant, although there was some indication that storage at 12.5 million sperm per milliliter was superior for storage periods of seven or more days.

Two field trials were conducted to determine the effect of sperm concentration on fertility, the first in the 1961 spring mating period and the second in the 1962 spring mating season. In the first experiment, split ejaculates from 12 bulls were diluted to contain either 12.5 million or 200 million sperm per milliliter. Both samples were used on the day after collection. The percentage of successful inseminations for semen stored at 12.5 million sperm per milliliter was 60.1% (2,858 inseminations) and 63.2% for semen stored at 200 million sperm per milliliter (2,591 inseminations). The difference of 3.1% in favor of storage at 200 million was significant (P < 0.025).

In the second experiment, split ejaculates from seven bulls were diluted in Caprogen to contain either 12.5 or 200 million sperm per milliliter. Seven days after collection sperm stored at 200 million sperm per milliliter was rediluted in Caprogen to contain 12.5 million sperm per milliliter. Both samples were used on the seventh day after collection. The percentage of successful inseminations for semen stored at 12.5 million sperm per milliliter was 52.5% (1,179 inseminations) and for semen stored at 200 million sperm per milliliter, 40.8% (1,207 inseminations). The difference of 11.7 percentage units in favor of storage in the diluted form was highly significant (P < 0.01).

At the dilution rates and in the diluents studied nitrogen saturation effectively protected sperm against dilution effects. It is presumed that the beneficial effect of nitrogen saturation is due to the reduction of oxygen tension in the diluting media, as the dilution rate effect can be simulated by increasing the oxygen tension in semen stored in concentrated form. However, measurement of actual O₂ tensions is required in the different methods of storage before this assumption can be confirmed.

Field trials suggest that storage in concentrated form and redilution immediately before use may give improved conception results in semen stored for short periods in an unsaturated diluent, but that storage in concentrated form is definitely detrimental for a storage period of a week in saturated diluents. These results also require further amplification. It is proposed, therefore, to compare conception rates of semen stored at varying dilution rates, in saturated and unsaturated diluents after varying storage periods.

REFERENCES


