Techniques and Results of Low Temperature Bull Semen Storage

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SINCE the first successful inseminations of dairy cattle with semen stored at very low temperatures were reported by Rowson and Polge in 1952, the technique has become well established in artificial breeding work. The temperature used is close to —79 degrees C (—114 degrees F) which happens to be the sublimation temperature of solid CO_2. The use of "deep-freeze" in this work is apt to be confusing as the deep-freeze refrigerators used for storing food run at from —15 to —20 degrees C, a temperature which is quite unsuitable for storing semen. Success has been claimed with semen at still lower temepratures, i.e., in liquid air at —190 degrees C and in liquid nitrogen at —196 degrees C. Apart from expense, it seems likely that the effective periods will be longer the lower the temperature, provided that the cooling techniques are satisfactory. For immediate consideration the important point is that the present standard practice of storing semen at —79 degrees C will permit successful insemination for at least a year, which is a tremendous advance on the maximum of three of four days storage with the diluents and temperatures now used for commercial artificial breeding.

It will be realised at once that the long storage possible with low temperatures will have special interest for artificial breeding in New Zealand, where one of the main obstacles to its widespread use is the very restricted breeding season. An effective storage of nine months would overcome the problem quite well. There are other very substantial advantages in being able to hold stocks of semen for long periods. The first that comes to mind is the possible provision of a nominated bull service for pedigree breeders. One of their main objections to artificial breeding at present is that they cannot choose any particular bull to mate to any particular cow. By keeping a bank of all bulls in the artificial breeding team in the main dairying districts, a choice could be made by pedigree breeders, with doubtless greater support from them in the future.

Another very real advantage of low temperature storage from the bull centre's viewpoint is the provision of service from minor breeds. That is for New Zealand for all breeds except Jerseys. For a great part of the year the daily demand for Fresians and Ayrshires is so small that one good service would last for a fortnight. The less frequent use of the few bulls available from the minority breeds would result in better quality material, and less handling of bulls. At present it frequently happens that a service has to be collected from a bull for two or three cows. Semen stored at low-temperatures can be used to import new stock, at a fraction of the cost of importing the bulls concerned. In addition very good safeguards against the introduction of serious cattle diseases can be set up, by holding batches of semen until donor bulls have been cleared of any possible risk of incubating a serious disease such as Foot and Mouth disease. It could conceivably happen during serious outbreaks of this disease in Europe that a demand for semen from a disease-free country such as this would arise.

As a final point, a demand has already developed for the storage of semen from valuable bulls against the day when their fertility will fall. Though perhaps not as important an aspect of this work as

those previously mentioned nevertheless of sufficient importance to individuals to make it worth their while to arrange storage for some months ahead.

This brief account of the applications of storing semen for long periods has been given so that when the details of the technique have been described the considerable advantages can be discussed in relation to the extra expense and labour involved in preparing, storing and distributing semen stored at low temperatures.

TECHNIQUE:

1. Semen collected in the usual way and subjected to the usual tests for appraising its quality.
2. A primary dilution is made immediately (within a few minutes of collection) with from 4 to 10 parts of standard Citrate-egg-buffer (equal parts of fresh egg-yolk and 3.5% Sodium Citrate $N_2C_6H_5O_7$. $2H_2O$ in glass distilled water) at 30 degrees C.
3. Slow cooling in ordinary refrigerator for 2-3 hours. When working with small quantities it is advisable to place the tube containing the diluted semen in a beaker with 300 mls of water at 30 degrees C. At the same time an equal quantity of 20% Glycerol in 3.5% Sodium Citrate solution is placed in the refrigerator.
4. When both solutions are at 5 degrees C, an equal volume of the 20% Glycerol solution is added to the primary dilution of semen making a final concentration of 10% Glycerol. The mixing must be done gradually, about 5 mls of the 20% glycerol solution being added to the semen every five minutes. Solutions must be held in the refrigerator when not being handled.
5. Leave the diluted semen in the refrigerator (5 degrees C) overnight or for 12 to 20 hours. The required number of glass ampoules should be labelled and placed in the refrigerator to cool ready for filling the next day. A half-gallon or gallon thermos flask, depending on the number of ampoules to be processed, is three-quarters filled with 95% alcohol and also placed in the refrigerator to cool to 5 degrees C.
6. After 12 to 20 hours equilibration with 10% Glycerol the diluted semen is pipetted in one ml amounts into the cooled glass ampoules, which are then sealed in a flame. It is important to keep the filled ampoules at 5 degrees C until the low-temperature process starts.
7. The sealed and labelled ampoules are placed in a wire basket with a wire mesh lid, and the basket is placed in the alcohol in the thermos flask which will be also at 5 degrees C. Sufficient alcohol should be provided to just cover the ampoules.
8. Small pieces of solid CO_2 are added to the alcohol in the flask, the rate being directed by the fall in temperature. Thermometers to read to —80 degrees C are available, and one can be used to check the rate of temperature drop. This should be one degree Centigrade every two minutes until the temperature has fallen to —10 degrees C. From this point the cooling rate can be increased by adding solid CO_2 more rapidly, the rate being now governed by the ebullition of the mixture. The whole process takes about 45 minutes.
9. When the temperature has fallen to —76 degrees to —79 degrees the ampoules can be transferred to the storage cabinet.
10. Semen must be maintained at this temperature until immediately before use, though a slight modification of this will be discussed later. If ampoules are to be transported they must be packed in CO_2 snow in a thermos flask.

11. Ampoules are prepared for insemination by placing them in a relatively large quantity of water at blood heat, or they may be thawed out by holding them under running cold water. Rapid thawing appears essential. The ampoules are etched with a small file, broken and the semen drawn up in an inseminating pipette and used in the usual manner.

It will be apparent to those familiar with the older method of semen dilution and storage that the low temperature process is more costly and time consuming. For those not familiar with the routine, the main differences are as follows:

a. Addition of glycerol.
b. Considerable labour in cleaning, labelling, filling and sealing individual doses in glass ampoules.
c. Cooling process more complicated.
d. Regular supplies of CO2 snow necessary for cooling, storing and dispatch.
e. Storage apparatus more costly. It is estimated that to store one ampoule for one year in a mechanical refrigerator running at —79 degrees C, allowing 20% depreciation would cost 1/-. Storage in a large cabinet requiring the constant addition of CO2 would cost 3d per ampoule per annum. The glass ampoules themselves cost 3d, and the same amount would cover the extra labour involved in cleaning and processing.
f. More skill and labour required at the sheds before the semen can be used.

The difficulties encountered in procession low temperature material are not as serious as was at first supposed, because in New Zealand the preparation of the material would be done at a time when the field work for ordinary techniques is almost at a standstill. Considerable improvements could also be affected in handling larger batches in the cooling process. Economy in storage may also be effected by storing semen in a relatively concentrated form and rediluting it further when thawed.

From the field side, although greater care is necessary in preparing the ampoules for insemination, it should not be beyond the ability of the type of man who is coming forward for training as an inseminator. The extra time taken over the work might be a serious disadvantage in the first three weeks of the season, particularly with new technicians. An examination of a fair cross section of the time-sheets of a number of technicians in several districts has shown that after the first three weeks the day's inseminations have been finished in well under five hours. However if as already mentioned, semen can be stored concentrated and then extended when thawed, this would cut down the work in the sheds as well as reducing the storage space in the low-temperature cabinets. This will be discussed again when dealing with the results.

It will be agreed that the potential benefit of low temperature storage in the application of artificial breeding in New Zealand greatly outweighs the mechanical difficulties at present still to be solved. The technique should be expanded with all our resources. It is as well to remember that reports from England show that the results from extensive low-temperature work are not quite as good as from the usual commercial method. During the present stage of very rapid expansion it is difficult to keep our conception rates as high as we would like, and they cannot stand any reduction at all, and at present there is a definite limit to what can be classed as research or investigational work in a commercial artificial breeding organisation.

RESULTS:

After preliminary laboratory work, a field trial was carried out by the Ruakura Animal Research Station in 1953. This covered nearly 1200 inseminations, the greatest proportion of which were done from

two bulls which had been selected as producing very good samples of semen. One bull which was responsible for 473 inseminations gave 49% success, the other most heavily used bull gave only 17% success from 386 inseminations. The average for commercial breeding that year was 45%. A few services from other bulls which had been collected and processed up to six months earlier showed as good results as that stored for only one month. This experience showed that (a) the semen from different bulls varied considerably in its freezing quality, a finding which has been confirmed by overseas workers, (b) it was not possible to pick these bulls from the laboratory examination of the semen samples and (c) the length of storage was not important if the material was inherently of good freezing quality.

Due to heavy expense in holding semen at low temperatures for many months, consideration was given to economy of storage space by holding semen at a more concentrate level and extending it further when thawed. As explained before this approach would also reduce the field work considerably.

A small trial carried out during the winter in the South Auckland area to test out the dilution-after-thawing principle gave very poor results 31% successful out of 29 inseminations. However in this work the semen was thawed out in the late afternoon, diluted and dispatched 80 miles for use the following day. This was a severe trial and in practice the length between thawing and redilution and insemination could be very much reduced. When this interval was reduced to less than six hours, that is material that was thawed out in the early morning and used rediluted before midday gave 40% success with 78 inseminations compared with 49% with 254 inseminations when the semen was used on the farm immediately after thawing.

These results were confirmed in another small experiment, where split samples were thawed, diluted and dispatched in the evening and compared with samples from the same batch thawed and sent out in the morning. The results from the evening dispatched, material up to 18 hours old, was 5% successful, and the morning samples 38% successful after 6 hours. One small batch gave 50% success with the morning treatment. When the delay between thawing and insemination was cut down to less than an hour by a technician thawing and rediluting in the sheds as he went round the result reached a very useful level, of just over 50%.

Nominated Service for Pedigree Breeders:

A start was made in 1954 with a nominated service for Pedigree breeders. This was restricted to the Hamilton area, as it was not considered advisable to place it further afield with the unsolved problems of distribution. It was fortunate that an experienced technician was available in this area to work solely on the nominated service, thus avoiding extra work for newly trained technicians which were operating the groups in the area.

To evercome the difficulty in providing a prompt service after the daily calls had come in the morning, the technician was provided with a gallon size thermos flask which contained a few ampoules from each of the popular bulls. This was returned to the centre every second day for replenishing the CO_2 lost, and for restocking the used ampoules. In the meantime a second thermos was sent out to keep up a continuity of service in the field. Farmers could thus phone their requirement directly to the technician. It soon became clear that the demand centred round a very few bulls. This type of arrangement would work well for overcoming the daily fluctuations in demand for grade cows. The percentage of insemination now used by pedigree breeders is not more than 4%, so it is hoped that a much greater

number of farmers will avail themselves of this service in future. In the area in which this scheme operated this past season, and which contained well above the average number of pedigree cows, only 208 cows were inseminated with a 46% success rate. One bull was responsible for 50% of the demand and his results gave 56% success. Another bull gave 47% conception rate a few weeks after he had died. The results though on small numbers seem encouraging enough to warrant increasing the scope of the service very considerably next season.

Importations of Bull Semen Stored at Low Temperatures:

A limited amount of work has been done with imported semen. Two batches were received from the Ministry of Agriculture's Centre at Reading through arrangements made by Professor Riddett. Some delays en route with possible disorganisation in the repacking with dry-ice schedule occurred with the first batch. The motility rating on arrival was low (15 to 25% motile). However some 38 cows were inseminated of which 14 held 36.8%. Included in this number were eight cows at Massey College of which 4 were in calf four months after insemination.

The second batch arrived without delay, and appeared much more active on arrival. Unfortunately the season had so far advanced that few ampoules could be used. Four cows were inseminated in three herds, all of which held to the first insemination. Although an attempt was made to hold over this material until the following spring, the remainder was lost due to an accident to the thermos containing the batch.

The gallon thermos flasks used to send out the frozen ampoules hold 100 ampoules, the cost of transport from Great Britain being in the region of £40. This aspect of low-temperature storage is certainly practicable, but the arrangements are so encompassed by regulations at both ends that it is a herd improvement measure that can be undertaken only by the stoutest hearts.

Although there has already been some demand for storage space for banking bull semen for individual farmers, we have not any results to report.

It seems, therefore, that the low-temperature storage technique is giving workable results, and should be extended both for routine grade cow inseminations and for a nominated pedigree service. The dilution after thawing modification though encouraging, needs more investigation before its use can be recommended on a large scale.

Discussion

Dr. McLEAN: We have had some experience with imported frozen semen. The motility appeared to be very poor though we had to use it.

Dr. JAMES: We have had the same experience, and though the material has shown a poor motility as judged by comparison with fresh semen the conception rates have been surprisingly good.

Mr. JEBSON: How are the ampoules sealed? Does the heat required have any effect, or are the corks used?

Dr. JAMES: The ampoules are sealed by heat. With a hot flame the process only takes a few seconds, and very little of the heat is conducted through the glass.

Mr. McFARLANE: In the Gisborne Veterinary Club we aim to give an eight months' service. We have found it costly because we order each day by phone, cartage is expensive, and wastage is high. Frozen semen would be very useful in isolated areas. What is the cost of dry-ice for storage on the spot?

Dr. JAMES: The cartage on dry-ice would be expensive if deep-freeze storage was attempted on a small scale. The principle of such storage is that we want to overcome the daily wastage problem. It looks at present that a special deep-freeze refrigerator is going to be cheaper to run than a dry-ice cabinet in New Zealand.

Mr. McKENZIE: Is freezing effective in controlling infections?

Dr. JAMES: **Trichomonas foetus** appears to be killed by deep-freezing, but bacteria are resistant. We have had some success with using antibiotics in frozen semen, so this may safeguard the position as far as venereal infections are concerned. We expect to be able to screen our bulls from such risks in the process of selection. Bacteria which are commonly found in bull semen but which are not associated with specific diseases do not appear to be killed by deep freezing.