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MOTILITY AND FERTILITY OF BOVINE SEMEN STORED AT -79°C AND -196°C

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SUMMARY

The percentage of motile spermatozoa in bovine semen frozen in ampoules and stored for 120 days at either −79°C in solid carbon dioxide-alcohol or −196°C in liquid nitrogen was compared. The percentage motile spermatozoa was significantly higher ($P < 0.01$) after 14 days' storage at −196°C than at −79°C. This difference was still apparent after 120 days' storage ($P < 0.01$).

The transference of semen from storage in solid carbon dioxide-alcohol to liquid nitrogen produced no change in the percentage of motile spermatozoa. However, when semen was transferred from liquid nitrogen storage to solid carbon dioxide-alcohol there was a decline in motility similar to that found with primary storage in solid carbon dioxide-alcohol.

Split batches of semen stored for 90 days in solid carbon dioxide-alcohol and liquid nitrogen were used for the insemination of cows. The solid carbon dioxide-alcohol stored semen was transferred to liquid nitrogen for field storage. The first insemination 60-90 day non-return rates were 70·9% for semen stored in solid carbon dioxide-alcohol and 73·5% for semen stored in liquid nitrogen.

I. INTRODUCTION

Many studies on post-thawing spermatozoal motility, metabolism and fertility of deep frozen semen stored at various temperatures have been reported.

Various workers (Larson and Graham 1958; Pickett, Fowler, and Cowan 1960; Pickett *et al.* 1960; Pickett, Martig, and Cowan 1961) have demonstrated that post-thawing spermatozoal motility is higher for semen stored in liquid nitrogen (LN) than for semen stored in solid carbon dioxide and ethyl alcohol (DI). Sullivan and Mixner (1962, 1963) also found that semen stored in LN maintained a higher metabolic level than semen stored in DI.

Macpherson (1960) reported that there was no significant difference in the percentage motility of semen stored for 3 months at −87°C maintained by mechanical refrigeration and at −196°C by LN. However, Etgen and Ludwick (1955) and Etgen *et al.* (1957) found that storage at a higher temperature (−79°C in DI) resulted in semen with a lower post-thawing motility than when stored at −96°C maintained by mechanical refrigeration.

Evaluations of the fertility of semen stored at various temperatures have been conducted by Larson and Graham (1958), Macpherson (1960), Pickett *et al.* (1960), Pickett, Martig, and Cowan (1961) and Stewart (1964). There is general agreement that higher 60–90 day non-return (N.R.) percentages have resulted with semen stored in LN than by other methods. However, only Pickett, Martig, and Cowan (1961) have been able to demonstrate a significant difference in favour of liquid nitrogen storage.

Over a period of 3 years (1964–1966), storage of deep-frozen semen at the Artificial Insemination Centre, Wacol, Queensland, and at the 22 sub-centres in the State was converted from DI to LN. In 1963, the 60–90 day non-return percentage for deep-frozen semen was 60·8 for 25,109 first inseminations (Singleton 1966); in 1966 it had increased to 68·3 for 52,948 first inseminations (Singleton 1968). This experiment was undertaken to determine whether this 7·5% increase in non-return percentage could have been due to at least in part to LN storage or to increased insemination efficiency resulting from the use of more manageable liquid nitrogen field equipment.

II. METHOD AND MATERIALS

Experiment 1.—Ten ejaculates of semen were obtained from five Australian Illawarra Shorthorn and five Jersey bulls routinely in use at the Artificial Insemination Centre, Wacol. Immediately after collection, the volume of semen and the spermatozoal motility were recorded. The percentage of live normal spermatozoa was determined by using 3% congo red, 5% nigrosine staining (Blackshaw 1958) and spermatozoal density by the spectrophotometric method (Salisbury *et al.* 1943). Based on these determinations the semen was diluted in a stepwise manner to 50 x 10^6 live normal spermatozoa per ml by an extender which was prepared by mixing together equal quantities of two fractions. Fraction A contained 50% egg yolk and 50% buffered sodium citrate. Buffered sodium citrate was prepared from 8 parts of 3% trisodium citrate, 1 part of 3·58% disodium hydrogen orthophosphate and 1 part of 1·56% sodium dihydrogen orthophosphate by volume in distilled water. Fraction B consisted of buffered sodium citrate to which 15% glycerol by volume and 2·5% fructose by weight had been added.

The semen was initially extended to 100 x 10^6 live normal spermatozoa per ml by dilution with fraction A. This diluted semen was cooled from 32°C to 5°C over a period of 2–3 hr. An equal volume of fraction B at 5°C was added when the diluted semen attained this temperature to complete extension to 50 x 10^6 live normal spermatozoa per ml. After gently mixing, the semen was hermetically sealed in 1·0 ml glass ampoules and allowed to equilibrate at 5°C for 15 hr. The ampoules of semen were then placed in a "Linde" BF 3–2 liquid nitrogen biological freezer and cooled at the rate of 3°C per min from 5°C to −15°C, then at 8°C per min from −15°C to −70°C, and finally cooled to −196°C. Half of the ampoules from each bull were stored at −196°C in a "Linde" LNR300 LN refrigerator and half at −79°C in vacuum flasks with DI.

The percentage of motile spermatozoa in the semen of each of the 10 bulls was evaluated. On each occasion two ampoules of semen were randomly selected from each bull's batch in the LN storage units and two ampoules from each bull's batch in the DI storage unit. The semen was evaluated immediately the semen was frozen and at 3, 5, 7, 10, 12, 14, 17, 19, 21, 24, 26, 28, 34, 44, 61, 72, 90 and 120 days post-freezing. On each occasion the 40 ampoules of semen were thawed in water at 5°C and ampoules were selected at random and scored for the percentage of motile spermatozoa. The experiment was terminated after 120 days as this was the maximum time semen was stored in DI before it was despatched to field inseminators.

Experiment 2.—After 90 days' storage in DI or LN, the method of storage for half the remaining ampoules of semen used in experiment 1 was reversed for 7 of the 10 bulls. Two ampoules of semen from each batch at each storage temperature were randomly selected and evaluated for the percentage motile spermatozoa on transfer and at 5, 10, 12, 15, 17 and 33 days post-transfer.

Experiment 3.—Eighteen ejaculates of semen were obtained from five Australian Illawarra Shorthorn and two Jersey bulls. All the semen collected on one day from a bull was considered as one ejaculate. The semen was processed as in experiment 1 and examined for the percentage of motile spermatozoa at the time of storage and after 21 and 90 days in storage. After 90 days' storage the semen stored in DI and LN was transferred to a field insemination unit containing LN and remained in this until used for insemination of cows. The fertility of the semen was assessed on the 60–90 day non-return to service percentages of the cows inseminated.

III. RESULTS

Experiment 1.—The relationship between the mean percentage of motile spermatozoa in the semen stored for varying periods up to 120 days in LN and DI is shown in Figure 1. The percentage motility of semen stored in both LN and DI declined rapidly over the first 20 days and thereafter declined at a slower rate. However, the rate of decline during the first 20 days was greater for semen stored in DI than in LN, which resulted in it having a significantly lower percentage of motile spermatozoa ($P < 0{\cdot}01$) at each sampling between 20 and 120 days.

Experiment 2.—There were no significant changes ($P > 0{\cdot}05$) in the percentage motile spermatozoa in semen which had been stored in DI for 90 days and then transferred to LN for 33 days. The reverse changes from LN to DI resulted in a decline in percentage spermatozoal motility similar to that found in experiment 1 with semen stored in DI. The relationships between post-thawing motility and time are shown in Figure 2.

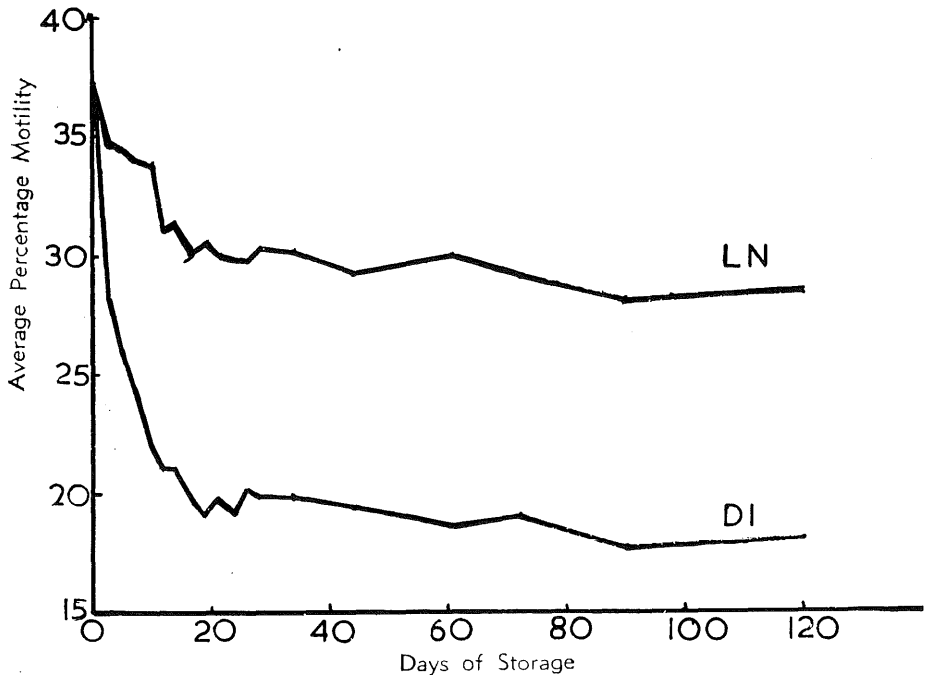

Fig. 1.—Relationship between the mean percentage of motile spermatozoa in ampoules of frozen semen stored in liquid nitrogen (LN) and solid carbon dioxide-alcohol (DI) and time.

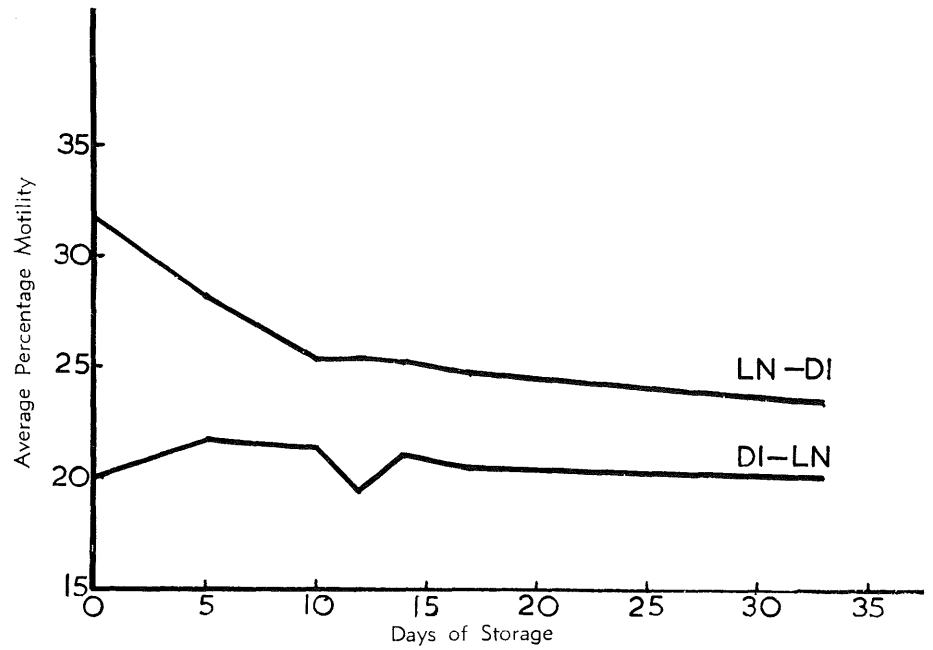

Fig. 2.—Relationship between the mean percentage of motile spermatozoa in ampoules of semen changed from liquid nitrogen (LN) to solid carbon dioxide-alcohol (DI) storage and from solid carbon dioxide-alcohol to liquid nitrogen storage.

Experiment 3.—A similar decline in the post-thawing motility of semen over the first 20 days in storage as found in experiment 1 was recorded in this experiment (Figure 3). Spermatozoal motility after 21 days' storage was significantly greater ($P < 0{\cdot}01$) for semen stored in LN than in DI. The mean 60–90 day non-return percentages for the semen stored in LN and DI were LN, $73{\cdot}5 \pm 1{\cdot}1$ for 947 first inseminations, and DI, $70{\cdot}9 \pm 1{\cdot}7$ for 876 first inseminations. The difference in non-return percentages due to storage temperatures was not significantly different at $P > 0{\cdot}05$. Two bulls showed a slight advantage in favour of storage at −79°C but in neither case was the difference significant ($P > 0{\cdot}05$). The other five bulls all showed a higher non-return percentage for semen stored in LN than in DI. In one bull this difference was significant ($P < 0{\cdot}01$).

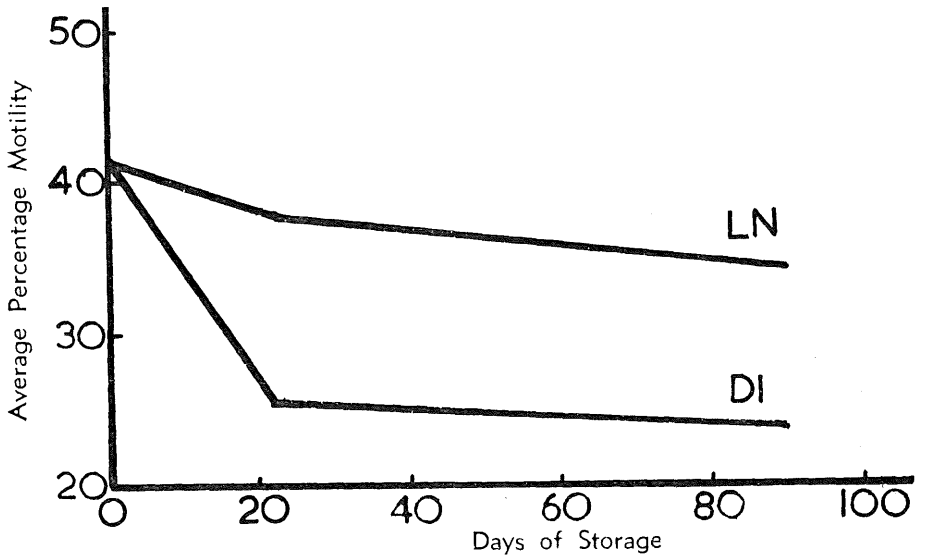

Fig. 3.—Relationship between the mean percentage motile spermatozoa in semen used in experiment 3 stored in liquid nitrogen (LN) and solid carbon dioxide-alcohol (DI) prior to insemination.

IV. DISCUSSION

The results of experiments 1 and 2, which show that a marked initial decline occurs in the motility of semen stored in DI and that motility of spermatozoa stored in LN is maintained at a significantly higher level ($P < 0{\cdot}01$) than when stored in DI, agree with those of most other workers (e.g. Larson and Graham 1958; Pickett, Fowler, and Cowan 1960; Pickett *et al.* 1960; Pickett, Martig, and Cowan 1961).

The higher 60–90 day non-return to service percentages of cows inseminated with semen stored in LN rather than DI is in agreement with the findings of Larson and Graham (1958), Macpherson (1960), Pickett *et al.* (1960), Pickett,

Martig, and Cowan (1961) and Stewart (1964). However, despite the motility differences that exist in favour of LN stored semen, the overall fertility was not significantly affected. In this experiment with an initial dilution before freezing of 50 x 10^6 live normal spermatozoa per ml, the number of motile spermatozoa per insemination remained greater than 10 x 10^6, which is the minimum number of live normal spermatozoa considered by Bratton, Foote, and Henderson (1954) to be required for optimum fertility. Where the initial dilution was higher or where the percentage of motile spermatozoa was less than that recorded in this experiment, the number of motile spermatozoa could more readily fall below this minimum number in DI than in LN storage. This could result in a significant difference in fertility. Storage of semen in LN may have the advantage that a greater dilution may be possible with semen from valuable sires. The higher percentage motility of spermatozoa stored in LN than DI should allow a greater dilution of semen than would be possible with DI storage.

There was an indication that the semen from some bulls shows individual differences in its reaction to storage temperature.

In addition to the motility decline on storage demonstrated in these experiments, LN provided a more convenient and safer method of storage in the field. It is suggested that greater temperature differential above the critical storage temperature for deep-frozen semen afforded by LN possibly reduces handling damage. These factors were probably important in the fertility improvement seen after the change from DI to LN in Queensland.

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