QUEENSLAND DEPARTMENT OF PRIMARY INDUSTRIES
DIVISION OF ANIMAL INDUSTRY BULLETIN No. 143

INFLUENCE OF ANTIBIOTICS AND NITROGEN ON POST-THAWING MOTILITY AND FERTILITY OF DEEP-FROZEN BOVINE SEMEN

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SUMMARY

Three treatments—the addition of penicillin (500 i.u./ml) and streptomycin (500 μg/ml); the flushing of ampoules with nitrogen; and the partial saturation of the diluent with nitrogen—were applied to a standard egg yolk diluent for deep-frozen bovine semen and compared with controls. No significant difference in post-thawing percentage of motile spermatozoa was observed ($P > 0{\cdot}05$). All three treatments resulted in a lower non-return rate to first inseminations than controls; however, the differences were not significant ($P > 0{\cdot}05$).

I. INTRODUCTION

The effects of including antibiotics, carbon dioxide and inert gases in liquid semen diluents have been reviewed by Almquist (1949, 1951), Wolberg (1959), Salisbury *et al.* (1960) and Kliewer and Wolberg (1962). Antibiotics in deep-frozen semen diluents have received little attention and reports by Dunn, Larson, and Willett (1953) and Erickson, Graham, and Frederick (1954) are conflicting. Similarly, reports on the effect of anaerobic conditions on post-thawing motility and fertility of deep-frozen bovine semen are also sparse and contradictory. Macpherson (1958) and Roussel, Patrick, and Kellgren (1962) reported an adverse effect from packaging under carbon dioxide, while Roussel, Patrick, and Kellgren (1962) and Steinbach and Foote (1964) found nitrogen flushing superior to or comparable with controls. Roussel *et al.* (1963) and Steinbach and Foote (1964) found no improvement in post-thawing motility resulting from the replacement of air in the diluent with argon and the flushing of ampoules with argon respectively.

The study reported here was designed to investigate the effects of the following three factors on the post-thawing motility and fertility of frozen bovine spermatozoa: the addition of antibiotics to the semen diluent, an increase in partial pressure of nitrogen in the semen diluent, and flushing the ampoules with nitrogen gas before filling with semen.

II. MATERIALS AND METHODS

Twelve first ejaculates, two from each of six bulls held at the Artificial Insemination Centre, Wacol, Queensland, were collected using an artificial vagina. The density of spermatozoa in the ejaculates was assessed by the spectrophotometric method described by Salisbury *et al.* (1943), using a Bausch and Lomb Spectronic 20 instrument.

The composition of the egg yolk glycerol citrate (EYC) semen extender used is shown in Table 1. The semen was diluted initially at 32°C in fraction A to a density of 100 x 10^6 live normal spermatozoa per ml. This fraction was then cooled to 5°C over a period of 2 hr, at which time an equal volume of fraction B was added to bring the final density to 50 x 10^6 live normal spermotozoa per ml. Diluted semen was sealed into 1 ml pyrex glass ampoules in a cold room set at 5°C, using a "Manning" model 21 ampoule filling and pull sealing machine. After an equilibration period of 18 hr the ampoules of semen were cooled from 5°C to −15°C at 3°C per min and from −15° to −196°C at 8°C per min, using a "Linde" BF3-2 biological freezer. The frozen ampoules were stored at −196°C in a "Linde" LNR300 liquid nitrogen refrigerator.

TABLE 1

COMPOSITION OF CONTROL DILUENT FOR DEEP-FROZEN BOVINE SEMEN (EYC)

Fraction A:	
Egg yolk	50% by volume
Buffered sodium citrate	50% by volume
Fraction B:	
Buffered sodium citrate	85% by volume
Glycerol	15% by volume
Fructose	0·25% by weight
Buffered Sodium Citrate:	
8 parts of 3·0% sodium citrate	
1 part of 1·56% sodium phosphate monobasic	
1 part of 3·58% sodium phosphate dibasic	

The antibiotic treatment (ANTI) was prepared by adding crystalline penicillin G (1,000 i.u./ml) and streptomycin sulphate (1,000 μg/lm) to fraction A of EYC. The addition of fraction B gave a final concentration of 500 i.u./ml and 500 μg/my of penicillin and streptomycin respectively in the diluent.

Nitrogen flushed ampoules (NF) were prepared by attaching a cylinder of compressed dry nitrogen to an auxiliary filling needle on the ampoule filling and sealing machine. Nitrogen was flushed into the ampoules through this needle at 15 p.s.i. pressure for 1 sec. The ampoules were machine-filled with extended semen 1 sec after flushing and sealed after a further period of 2 sec. Nitrogen saturated extender (NB) was prepared by bubbling nitrogen through fraction B of the EYC diluent for 20 min just prior to its addition to fraction A. Attempts to

also bubble nitrogen through fraction A were abandoned due to excessive frothing of the egg yolk. Semen was extended in the three diluents (ANTI, NF and NB), equilibrated, frozen and then stored in the same manner as previously outlined for EYC.

Bi-weekly estimates of post-thawing percentage motile spermotozoa were made on all four treatments for 6 weeks' post-freezing. Two ampoules of each treatment were used at each sampling period and the mean of the two estimations recorded. Estimations on thawed semen were made microscopically at X320 magnification, using a coverslip and a glass slide warmed to 32°C by a stage incubator. The identity of samples was unknown to the assessor.

Semen from the 12 split batches was used for insemination of cows to compare the fertility of controls with semen prepared by the three treatments. Two technicians performed the inseminations; each used control semen and semen diluted in ANTI, NF and NB for alternate cows. Ampoules of semen were labelled with a code so that their origin was unknown to the technicians.

III. RESULTS

The mean motility estimations on the 12 first ejaculates extended in EYC, ANTI, NF and NB diluents, for each bi-weekly sampling period during post-freezing at −196°C, are shown as percentages in Table 2. The average percentage of motile spermatozoa in ampoules sampled throughout the 6 weeks' storage post-freezing were as follows: EYC 28·4; ANTI 28·2; NF 28·1; and NB 28·2. Analysis of data showed no significant difference between any of the four treatments ($P > 0{\cdot}05$).

The number of first inseminations and the 60–90 day non-return rate with split ejaculate batches of the control and three treatments under test were as follows:—EYC: 305, 70·2%; ANTI: 338, 66·6%; NF: 284, 63·7%; NB: 272, 68·0%. The difference in the percentage of non-returns was not significantly different between controls and any one of the three treatments ($P > 0{\cdot}05$).

IV. DISCUSSION

With deep-frozen semen Dunn, Larson, and Willett (1953) showed that the addition of penicillin and streptomycin to the diluent did not significantly affect post-thawing motility estimations or fertility. The results reported here agree with these findings. The improved post-thawing motility recorded by Erickson, Graham, and Frederick (1954) following the inclusion of penicillin (500 i.u./ml) and streptomycin (500 μg/ml) was not observed in this study.

Roussel, Patrick, and Kellgren (1962) and Steinbach and Foote (1964) observed no significant improvement in post-thawing motility or fertility of semen following nitrogen flushing of ampoules. The results reported here agree with

TABLE 2

MEAN ± STANDARD ERROR OF THE PERCENTAGE MOTILE SPERMATOZOA IN FOUR DILUENT TREATMENTS STORED AT −196°C FOR 6 WEEKS

Diluent	Days of Storage											
	4	7	11	14	18	21	25	28	32	35	39	42
EYC	29·7±1·9	30·3±1·9	29·8±2·0	26·8±2·6	30·3±1·8	30·8±1·7	28·9±2·4	28·1±2·4	26·3±2·5	25·7±2·0	27·7±1·9	26·8±1·9
ANTI (EYC + antibiotics)	29·3±2·2	30·0±1·9	29·1±1·9	27·7±2·3	26·1±1·9	29·9±1·8	26·0±2·8	27·9±2·9	27·4±2·3	25·2±2·9	30·8±2·2	28·8±2·3
NF (EYC with nitrogen flushed ampoules) ..	30·7±1·4	28·0±1·8	29·6±1·5	27·4±1·9	27·5±1·8	28·5±1·4	27·9±2·8	28·5±2·5	27·1±2·5	27·0±1·6	27·2±1·1	27·5±1·8
NB (nitrogen saturated EYC)	30·2±2·3	27·0±2·2	28·2±1·7	28·8±2·0	28·3±2·0	29·5±1·6	25·8±1·7	26·2±3·2	28·2±3·1	27·5±2·0	30·7±1·5	27·9±2·0

these findings and in addition show that no significant improvement in either post-thawing motility or fertility of deep-frozen semen was obtained by nitrogenation of the diluent.

The results of this study indicate that the inclusion of antibiotics in the deep-frozen diluent had no significant effect on fertility. Accordingly, where the bacterial control afforded by penicillin and streptomycin is considered warranted, it appears they can be safely included at levels of 500 i.u./ml and 500 μg/ml respectively.

V. ACKNOWLEDGEMENTS

The assistance received from Messrs. G. W. Legg, W. B. Hornbuckle and R. Neiper of the Department of Primary Industries, Brisbane, in the collection and processing of semen and in the insemination of cows is gratefully acknowledged.

REFERENCES

ALMQUIST, J. O. (1949).—A comparison of penicillin, streptomycin and sulphanilimide for improving semen from relatively infertile bulls. *J. Dairy Sci.* 32:722.

ALMQUIST, J. O. (1951).—A comparison of penicillin, streptomycin and sulphanilimide for improving the fertility of semen from bulls of low fertility. *J. Dairy Sci.* 34:819.

DUNN, H. O., LARSON, G. L., and WILLETT, E. L. (1953).—The effects of freezing bovine spermatozoa in extenders containing antibacterial agents. *J. Dairy Sci.* 36:728.

ERICKSON, W. E., GRAHAM, E. F., and FREDERICK, E. C. (1954).—The effect of antibiotics, levels of glycerol, and rates of freezing on revival rate of bovine spermatozoa in yolk citrate and milk extenders. *J. Dairy Sci.* 37:651.

KLIEWER, R. H., and WOLBERG, F. B. (1962).—Gas pressure technique for preservation of bovine spermatozoa. *J. Dairy Sci.* 45:533.

MACPHERSON, J. W. (1958).—The fertility of frozen bovine semen packaged under carbon dioxide. *J. Am. Vet. Med. Ass.* 133:431.

ROUSSEL, J. D., PATRICK, T. E., and KELLGREN, H. C. (1962).—Effects of nitrogen and carbon dioxide on livability and fertility of frozen bovine semen. *J. Dairy Sci.* 45:527.

ROUSSEL, J. D., PATRICK, T. E., KELLGREN, H. C., and SHELWICK, J. O. (1963).—Influence of nitrogen and argon gases on past-thawing motility, laboratory stress tests and fertility of frozen bovine spermatozoa. *J. Dairy Sci.* 46:1278.

SALISBURY, G. W., BECK, G. H., ELLIOT, I., and WILLETT, E. L. (1943).—Rapid methods for estimating the number of spermatozoa in bull semen. *J. Dairy Sci.* 26:69.

SALISBURY, G. W., VANDEMARK, N. L., LODGE, J. R., and GRAGLE, R. G. (1960).—Inhibition of spermatozoa metabolism by p CO_2, pH and K ion and antibacterial compounds. *Am. J. Physiol.* 198:659.

STEINBACH, J., and FOOTE, R. A. (1964).—Effect of catalase and anaerobic conditions upon the post-thawing survival of bovine spermatozoa frozen in citrate and tris buffered yolk extenders. *J. Dairy Sci.* 47:812.

WOLBERG, F. B. (1959).—Gas pressure used to preserve semen. *Mod. Vet. Prac.* 40:51.

(Received for publication June 3, 1969)

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