OBSERVATIONS ON THE VIABILITY OF *TRICHOMONAS FOETUS* DURING THE PROCESS OF FREEZING TO −79° C. AND THAWING IN THE PRESENCE OF GLYCEROL

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The technique of storing bull semen at −79° C. in the presence of glycerol has become widely practised in recent years, and the usefulness of the method was increased when Joyner (1954) showed that *Trichomonas foetus* could be eliminated from infected semen by subjecting it to a freezing process similar to that used for the preservation of spermatozoa. Exceptional sensitivity of this flagellate to freezing was confirmed by McEntegart (1954), who found that while other species of *Trichomonas* survived the freezing and thawing process *T. foetus* was killed.

A number of other workers, however, have reported the recovery of *T. foetus* after freezing to −79° C., but in every case the methods used differed from that followed by Joyner (1954). Accordingly, it was evident that the freezing method could not be relied upon to eliminate the parasites until it was known which modifications in technique permitted the protozoa to survive.

The first experiments described in this paper were carried out to confirm that *T. foetus* in semen did not survive freezing and thawing in egg-yolk citrate as originally described (Joyner, 1954), with the added precaution of assessing the viability of the spermatozoa before and after freezing, by the eosin vital staining technique.

From the literature it seemed likely that some modifications in the suspending medium might permit the survival of the protozoa. Thus, in their freezing experiments from which *T. foetus* was recovered, Levine & Marquardt (1955) used cysteine-peptone-liver infusion-maltose (C.P.L.M.) medium with 5–10% glycerol, and Leidl & Mahrla (1954) used serum broth with 10% glycerol. McWade & Williams (1954) demonstrated the survival of *T. foetus* in a milk semen diluent containing 7% glycerol. All these suspending media differed from the egg-yolk-citrate semen-diluent with 10% glycerol in which the trichomonads did not survive (Joyner, 1954). The second series of experiments was, therefore, designed to demonstrate the effects of different diluents and varying concentrations of glycerol using trichomonads from cultures and preputial washings.

METHODS

The trichomonads were derived from preputial washings from a heavily infected bull and from stock cultures of the Belfast strain of *T. foetus*. The viability of the parasites was assessed by inoculation into glucose-broth-serum medium containing streptomycin and penicillin as previously described (Joyner, 1954).

The freezing process was identical with that used in the previous investigations (Joyner, 1954), except that the glycerol was added gradually over a period of 1 hr.

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This modification was adopted because there is some evidence that the rapid addition of glycerol can be detrimental to spermatozoa (Rowson, personal communication). Practical experience in an Artificial Insemination Centre where the modified method has been in continuous use confirms that it consistently permitted a high degree of sperm survival.

The trichomonads were separated from the washings or culture medium by centrifugation and resuspended in semen or seminal plasma. This suspension was mixed with nine volumes of diluent at room temperature. Twice the required final concentration of glycerol was prepared separately in the same diluent or the corresponding buffer solution and equal volumes of the two mixtures were placed in a refrigerator at + 5° C. for 3–4 hr. The glycerol was then gradually added in at least six portions over a period of 1 hr. The mixture was left at + 5° C. overnight and then distributed into cooled ampoules. The ampoules were heat sealed and immersed in alcohol at + 5° C. and the temperature reduced to − 10° C. at the rate of 0·5° C. per minute by the gradual addition of solid carbon dioxide. The rate of freezing was then accelerated to approximately 3·0° C. per minute until a temperature of − 79° C. was reached. After at least 20 hr. at this temperature the ampoules were thawed by immersion in warm water at 37° C. Samples of 0.5 ml. were removed for cultural tests for the presence of viable trichomonads.

The diluents used were as follows:

(1) Culture medium composed of glucose phosphate broth containing 7·0% horse serum.

(2) A mixture of equal volumes of egg-yolk and citrate buffer consisting of 3·92% sodium citrate solution adjusted to pH 6·7 (Wellcome).

(3) A mixture of equal volumes of egg-yolk and phosphate buffer consisting of 2% $Na_2HPO_412H_2O$ in 0·2% KH_2PO_4 (Campbell & Edwards, 1954). When using these two diluents the glycerol was diluted in the buffer solution only so that in the final mixture the egg-yolk was diluted 1:4.

(4) Pasteurized skimmed milk prepared by heating skimmed fresh milk at 96° C. for 15 min.

The eosin staining technique was used in exps. I and II (Table 1) in order to determine the survival rate of spermatozoa before and after freezing and thawing. The stain consisted of 1 g. eosin Y (Gurr) dissolved in 30 ml. citrate buffer pH 6·7 (Wellcome). 1·0 ml. of diluted semen was placed in a water-bath at 37° C. and after 5 min. 0·25 ml. of the stain previously warmed to 37° C. was added. The stain and semen were thoroughly mixed and allowed to stand in the water-bath for a further 5 min. A drop of the mixture was smeared on a warm slide also at 37° C. The smear was dried rapidly on a hot plate. At least three hundred spermatozoa were examined under a microscope, and the percentage of living cells which failed to absorb the stain was determined. The technique has been used by one of the authors during the freezing of large numbers of semen samples at a commercial Artificial Insemination Centre where it has been found to be a reliable indicator of deterioration in the quality of semen.

RESULTS

Freezing in viable semen

Trichomonads obtained from an infected bull were concentrated in 1 ml. seminal plasma and added to 2 ml. fresh semen at 20° C. The mixture was diluted in 1 in 10 egg-yolk citrate and the sperm viability estimated by the eosin vital staining technique. The mixture was divided into three equal portions, each equilibrated with 5, 7·5 and 10 % of glycerol, respectively. Samples were removed and inoculated into culture medium for the detection of trichomonads and the remainder was frozen in the normal way. After thawing, the viability of the spermatozoa was assessed and cultural tests were made for the presence of trichomonads.

The results of two such experiments are recorded in Table 1. In both cases adequate sperm survival was obtained with 7·5 and 10 % glycerol. Although the trichomonads survived the equilibration process, living organisms could not be detected after freezing and thawing.

Table 1. *The survival of* Trichomonas foetus *and bull spermatozoa after freezing to* −79° *C. and thawing in different concentrations of glycerol in egg-yolk-citrate diluent*

Experiment	Final dilution of semen	Estimated final concentration of trichomonads (per ml.)	Initial sperm viability (% alive)	Concentration of glycerol (%)	Final sperm viability after thawing (% alive)	Presence of viable trichomonads	
						Before freezing	After freezing
I	1/20	12,000	82	5	57·6	+	neg.
				7·5	67·5	+	neg.
				10	63·2	+	neg.
II	1/10	30,000	61·2	5	31·1	+	neg.
				7·5	53·8	+	neg.
				10	39·0	+	neg.

The effect of variation in glycerol concentration in different diluents

In the experiments to demonstrate the effects of different diluents and varying concentrations of glycerol, the trichomonads were derived both from preputial washings and from cultures.

The protozoa were concentrated in seminal plasma to contain either 5,000,000 trichomonads per ml. from cultures or 500,000 trichomonads per ml. from preputial washings. Before freezing, the infected seminal plasma was diluted 1 in 5 with the appropriate diluent and an equal volume of the diluted glycerol was added in accordance with the standard freezing procedure.

For the detection of viable trichomonads after freezing and thawing, samples of 0·5 ml. were added to culture medium. With organisms derived from preputial washings, additional tests were carried out to assess the viability of the trichomonads before freezing.

The results recorded in Table 2 show that in these experiments the organisms derived from preputial washings appeared to be particularly sensitive to egg-yolk-citrate diluent because in contrast to the experiments recorded in Table 1 the parasites suspended in this mixture were dead even before freezing. It was also

Table 2. *The effect on trichomonads of freezing and thawing in the presence of varying concentrations of glycerol in different diluents*

		Trichomonads from preputial washings			Trichomonads from cultures	
			Results of cultural tests for presence of trichomonads			
Percentage of glycerol	Diluent	Experiment	Before freezing, after 18–20 hr. at +5° C. in presence of glycerol	After freezing to −79° C. and thawing	Experiment	Results of cultural tests for presence of trichomonads after freezing to −79° C. and thawing
5	Culture medium	A	+	neg.	I	+
		B	+	neg.	II	neg.
	Egg-yolk citrate	A	neg.	neg.	I	+
		B	neg.	neg.	II	+
	Egg-yolk phosphate	A	+	neg.	I	+
		B	+	+	II	+
	Milk	A	+	neg.	I	+
		B	+	+	II	+
7·5	Culture medium	C	+	neg.	I	neg.
		D	+	neg.	II	+
		E	+	neg.		
	Egg-yolk citrate	C	neg.	neg.	I	+
		D	neg.	neg.	II	+
		E	neg.	neg.		
	Egg-yolk phosphate	C	+	+	I	+
		D	+	neg.	II	+
		E	+	+		
	Milk	C	+	+	I	neg.
		D	+	+	II	+
		E	+	+		
10	Culture medium	F	+	neg.	I	+
		G	.	neg.	II	neg.
	Egg-yolk citrate	F	neg.	neg.	I	neg.
		G	.	neg.	II	neg.
	Egg-yolk phosphate	F	neg.	neg.	I	+
		G	.	neg.	II	+
	Milk	F	+	+	I	neg.
		G	.	neg.	II	neg.

evident that most trichomonads survived the freezing process in the presence of 7·5% glycerol in egg-yolk phosphate or milk. The trichomonads from cultures appeared to be able to tolerate a wider range of conditions—7·5 or 5·0% of glycerol in all the diluents tested.

The toxicity of 10% *glycerol for trichomonads in different diluents*

The apparent sensitivity of trichomonads from preputial washings to glycerol in egg-yolk citrate mixtures was of particular interest because it offered an explanation of previous results. This phemonenon was therefore investigated in greater detail by suspending equal numbers of trichomonads from cultures and preputial washings in various media with or without 10% glycerol, the viability of the parasites being tested at intervals.

Table 3. *The toxicity of* 10% *glycerol for trichomonads in different diluents. Results of cultural tests for the presence of viable trichomonads*

	Origin of trichomonads									
	Culture					Preputial washing				
	Glycerol									
Time after addition of trichomonads (hr.) ...	4	20	28	54	76	4	20	28	54	76
Diluent:										
Milk	+	+	+	+	neg.	+	+	+	neg.	neg.
Egg-yolk phosphate	+	+	neg.	neg.	neg.	+	+	neg.	neg.	neg.
Egg-yolk citrate	+	neg.	neg.	neg.	neg.	+	neg.	neg.	neg.	neg.
	No Glycerol									
Time after addition of trichomonads (hr.) ...	4	20	28	54	76	4	20	28	54	76
Diluent:										
Milk	+	+	+	+	+	+	+	+	+	+
Egg-yolk phosphate	+	+	neg.	+	+	+	+	+	+	+
Egg-yolk citrate	+	+	+	+	neg.	+	+	+	+	+

Trichomonads from the two sources were suspended in saline and the number of organisms adjusted so that both suspensions contained 450,000 trichomonads per ml., and 0·2 ml. of this suspension was added to 0.8 ml. of each of the egg-yolk citrate, egg-yolk phosphate or milk diluents. After 3 hr. at +5° C. a further 1 ml. of the appropriate diluent was gradually added. In one series the second addition contained 20% of glycerol, making the final concentration 10%, and in the other series the diluent alone was added. Two series of mixtures were thus obtained with trichomonads derived from cultures and from preputial washings. Cultures were prepared from samples of 0·2 ml. removed at intervals from the suspensions stored at +5° C.

From the results recorded in Table 3 it is evident that the trichomonads remained alive for 54 to 76 hr. in the plain diluents, but in the presence of glycerol their survival in egg-yolk citrate was markedly curtailed. The longest survival in the presence of glycerol was observed in milk.

DISCUSSION

The conflicting literature concerning the survival of *T. foetus* after freezing and thawing has been discussed by Levine & Marquardt (1955), and the suggestion has been made that several differences in the techniques employed by the various authors may account for the different results. Among the factors which have been considered are: the speed of freezing, the actual numbers of organisms frozen and the proportion surviving, strain differences and the nature of the suspending medium.

The present study has been conducted with a view to application in the field of bovine artificial insemination, and only variations within the range of conditions known to permit adequate sperm survival have been considered. The most important factor within this limitation which could be varied was the suspending medium. It is evident that the nature of the diluent has a profound effect upon the sensitivity of the organisms to glycerol. 10% glycerol does not appear to be markedly toxic for trichomonads except in the presence of citrate buffer. Whenever an attempt is made to control the transmission of trichomoniasis by the application of this method egg-yolk citrate is the only diluent which can be used.

In the previous study (Joyner, 1954) there was some evidence that trichomonads derived from preputial washings were more sensitive to the freezing and thawing process than parasites derived from cultures. This is also suggested by the observations recorded in Table 2 where many more survivals were recorded when the trichomonads were derived from cultures, but in both these series of observations the numbers of organisms derived from cultures greatly exceeded those derived from preputial washings. The experiments of Levine & Marquardt (1955) have shown that even when conditions are most favourable, a considerable proportion of the organisms are killed during the freezing and thawing process. In experiments where the numbers of organisms differ widely, the chances of detecting viable organisms after thawing are greatest when the number of organisms frozen is high. This probably accounts for the apparent differences in survival rates when organisms from the two sources were used. It was only when the numbers of organisms were equalized, as in the experiment recorded in Table 3, that their sensitivity to glycerol could be reliably compared.

It is evident that *T. foetus* will survive freezing and thawing in the presence of glycerol in the culture medium described above or in the milk or egg-yolk phosphate diluents. The most suitable concentration of glycerol, to obtain maximum recovery, is approximately 7·5%. The same freezing process applied to the parasites suspended in egg-yolk citrate in the presence of 10% glycerol ensures their destruction.

From the results given in Table 3 it is evident that citrate ions alone are not excessively toxic, but in their presence the trichomomads appear to be sensitive to the effects of glycerol.

Trichomonads have been found on some occasions to survive up to 20 hr. exposure to 10% glycerol in egg-yolk citrate. In the experiments recorded in Table 1 the parasites survived overnight storage at 5° C., but in Tables 2 and 3 they did not survive this treatment. This variation appears to be similar to that noted by

Levine & Marquardt (1955) between the results obtained in different experiments. It should be noted that after freezing and thawing in this diluent the results of the viability tests have been consistently negative.*

Smith (1954) has reviewed the literature on the freezing of living cells and points out that different cells require different treatments for their survival. The above work provides an example where one and the same freezing process permits the survival of one type of cell (the spermatozoa), but destroys the other (the trichomonads). If, however, a different diluent is used, then both may survive.

SUMMARY

1. It has been confirmed that *Trichomonas foetus* fails to survive freezing and thawing in the presence of 10% glycerol in egg-yolk-citrate diluent. Under the same conditions adequate sperm survival was demonstrated.

2. Trichomonads were particularly sensitive to the toxic effects of glycerol when suspended in egg-yolk citrate.

3. Trichomonads will survive freezing and thawing when suspended in other diluents such as egg-yolk phosphate or milk.

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* During the course of the preparation of this work for publication Blackshaw & Beattie (*Aust. vet. J.* **31**, 214, 1955) have reported the survival of *T. foetus* after freezing and thawing in 5 and 7·5% glycerol in a variety of media including in some cases a citrate buffer. This was achieved by reducing the period of exposure to glycerol by freezing immediately after this substance had been added.

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