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Preservation of Rabbit Spermatozoa: Ethylene Glycol *vs* Glycerol for Frozen Semen.* (28511)

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Live young have been produced by female rabbits inseminated with previously frozen semen(1). The semen diluent contained 8% glycerol (G). For practical use, however, a higher fertility rate than first reported was needed. It has been demonstrated(2-4) that for the rabbit, ethylene glycol (EG) may be more satisfactory than glycerol for sperm preservation.

Two alternative procedures for incorporating G into a semen diluter prior to freezing have been: (a) initial semen dilution with a nonglycerolated diluter at room temperature, subsequent cooling to 5°C, and then stepwise addition of G at 5°C; or (b) initial (all-at-once) incorporation of G in the diluter with semen dilution performed at 37°C and subsequent cooling to 5°C. There are contradictory reports(5-9) based almost exclusively on the bovine as to the relative effectiveness of these two methods.

In seeking to improve the initial diluter, we set up 4 experiments to evaluate the effects of substituting EG for G and to obtain some evidence on the influence of the method of glycerol addition on survival of frozen rabbit spermatozoa.

Materials and methods. Semen was collected in an artificial vagina from primarily strain III (New Zealand White) bucks of The Jackson Laboratory rabbit colony. Semen was collected 5:30-7:30 AM, when distracting noises and influences were minimal. It was then evaluated for motility (rated 0-5), concentration (haemocytometer count), and live sperm (number of live sperm in 100 counted, using the differential stain fast green FCF and Eosin B as reported by Mayer *et al.*[10]). Samples having less than either 50% live sperm or 100 million/cc were eliminated from treatment comparisons on the grounds that they were of such inferior quality that results might be biased. The split sample technique was used with 0.15 cc undiluted semen/treatment in all comparisons. Immediately after collection and initial dilution all semen was cooled slowly to 5°C during the course of 1 hour. It was frozen at 3:30 PM the same day by the procedure of Fox(1) and stored at –90°C in a 2-phase mechanical freezer. The basic diluters used in all the experiments are listed in Table I.

Exp. 1. Semen was initially diluted 1:5 at 20°C with a nonglycerolated diluter and 3 hours later 1:2 in a stepwise manner at 5°C with the same diluter, having either 16% EG

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TABLE I. Diluters Used in 4 Experiments.

Constituents	Constituent concentrations in Exp. No.* 1	2	3	4
Egg yolk	20 cc	20 cc	20 cc	20 cc
Sodium citrate dihydrate	1.160 g	1.160 g	1.160 g	1.160 g
Dextrose	—	—	1.041 g	.240 g
l-Arabinose	.240 g	.240 g	—	—
Glycine	.7496 g	.7496 g	.7496 g	.7496 g
Sodium bicarbonate	.1680 g	.1680 g	—	.1680 g
Potassium chloride	.0320 g	.0320 g	—	.0320 g
Citric acid	.0100 g	.0100 g	—	.0100 g
Glycerol	0 cc, 16 cc	—	8 cc	8 cc
or	or		or	or
Ethylene glycol	0 cc, 16 cc	(5°C) 0 cc 16 cc (37°C) 8 cc	8 cc	8 cc

* Distilled H_2O—enough to make total volume of 100 cc in all 4 experiments.

or G(1). Equilibration time was 5 hours, followed by freezing and storage for 48 hours.

Exp. 2. Two methods of incorporating EG into the final diluter were evaluated. The method of adding EG in Exp. 1 was compared with incorporating 8% EG in the original diluter and maintaining diluent and semen at 37°C until a 1:10 dilution was made. Equilibration times were 5 and 8 hours respectively. From limited (unpublished) data the all-at-once addition of EG at 37°C appeared to be more satisfactory than at 20°; therefore, 20°C was not used in this experiment. The paired samples were then frozen simultaneously to avoid introducing possible variations due to differences in freezing. Storage time was 48 hours.

Exp. 3 and 4. Comparisons were made of semen diluted 1:10 at 37°C with an extender containing either 8% EG or G. The diluted semen was stored frozen for 3 months. The basic diluter in Exp. 4 was different from that used in Exp. 3 in order to determine whether or not the relationship of EG to G would vary due to a possible interaction effect with the diluter as a whole.

The semen in all experiments was thawed at 5°C, then warmed in a 37°C water bath for 5 minutes. After thawing, all semen was further diluted 1:2 to reduce glycerol (or substitute) concentration from 8% to 4%. This serves to revive the dormant sperm population from an inhibitory effect of G or EG on motility and allows a valid evaluation with a live-dead stain. Mixner and Saroff(11), using bovine semen, showed that as the level of glycerol in the diluter exceeded 4% the percentage of live to motile sperm decreased disproportionately.

Both members of each paired comparison were thawed, warmed, and examined simultaneously by the same observer. On this basis, paired t-tests were employed on final live sperm and motility ratings(12).

Results. The data of Exp. 1, 3, and 4 show (Table II) that there is uniform and significant improvement in sperm viability and motility when EG is substituted for G in any of the diluters (Table I). The increase in the number of unstained (presumably live) sperm is 10.3, 11.1, and 9.2 per hundred, respectively, with the corresponding changes in motility ratings being 1.0, 1.5, and 1.8 units. The mean difference in the proportion recovered following freezing (weighted for the number of samples involved) is 14% more live sperm and 28.25% greater motility for EG than for G.

In Exp. 2 (Table II) a significant improvement of 6.5 more live sperm was found when 8% EG was in the original diluter, with dilution made at 37°C and subsequent cooling. This is in comparison with initial dilution with a nonglycolated diluter, followed by stepwise addition at 5°C of equal parts of a 16% EG diluter. The second dilution made the final concentration of EG 8%. The difference in motility appeared to be in favor of EG addition at 37°C compared with 5°C, but was not significant.

TABLE II. Effect of Treatment on Sperm Viability and Motility After Storage at −90°C.

Exp	Criterion[2]	No. samples	Treatments[1] A	Treatments[1] B	Mean initial rating[2] I	Mean rating after storage[2,3] A	Mean rating after storage[2,3] B	Proportion recovery A/I	Proportion recovery B/I
1	No. live	23	EG	G	75.1	23.7	13.4	.32	.18
	Motility	—	—	—	4.5	3.6	2.6	.80	.59
2	No. live	25	37%	5%	74.0	39.0	32.5	.53	.44
	Motility	—	—	—	4.4	3.6*	3.4*	.82	.77
3	No. live	12	EG	G	79.0	44.6	33.5	.56	.42
	Motility	—	—	—	4.6	3.9	2.4	.85	.52
4	No. live	11	EG	G	62.6	31.6*	24.9*	.50	.40
	No. live	10	—	—	63.6	33.3	24.1	.52	.38
	Motility	11	—	—	4.2	3.0	1.2	.71	.29

[1] G = glycerol; EG = ethylene glycol; 5°C refers to stepwise addition of EG after semen has cooled to 5°C; 37°C refers to initial dilution of semen at 37°C with EG incorporated in diluter.

[2] Units for treatment comparisons are: No. live = number unstained sperm in 100 counted using a differential live dead stain; Motility = units based on an arbitrary 0-5 rating system.

[3] Differences between A and B: differences between starred (*) values not statistically significant ($P = .05$). All other differencs significant ($.025 > P \geq .001$).

The data comparing 4% *vs* 8% (EG or G) (Table III) show a significant increase in both the proportion of live sperm and the motility ratings in all cases when concentration of EG or G was reduced from 8 to 4%. The mean differences (weighted by number of samples) are for EG 25 more live sperm/100 and 2.53 units increase in motility; for G the corresponding values are 9 more live sperm/100 and 1.36 units increase in motility. It must be noted that the number of samples in the proportion live is very small.

Discussion. The use of EG in frozen semen diluters shows marked superiority to G in both the proportion of live sperm and motility. Several factors may account for the difference. It has been reported(13-16) that the protective action exerted during the freezing process is due to the effect on electrolytic concentration, apparently *via* simple colligative action. Differences in protective action between EG and G may therefore be due to the greater numbers of EG molecules present, the molecular weights being 62 and 92 respectively. Differential permeability between EG and G could be a factor, either due to difference in molecular size or to some property other than size. Another possibility

TABLE III. Effect of Level of Alcohol in the Diluent on Motility and Differential Staining of Sperm After Storage.

Exp	Diluent[1]	Criterion[2]	No. samples	Mean rating[2,3] After dilution (4% EG or G)	Mean rating[2,3] Before dilution (8% EG or G)
1	G	No. live	5	11.8	2.8
		Motility	24	2.6	1.2
	EG	No. live	5	25.2	0
		Motility	24	3.5	1.0
2	EG (5°C)	"	18	2.9	.2
	EG (37°C)	"	18	3.3	.7
3	EG	"	12	3.9	.9
	G	"	12	2.4	.8
4	EG	"	11	3.0	1.3
	G	"	11	1.3	.3

[1] G = glycerol; EG = ethylene glycol; 5°C refers to stepwise addition of EG after semen has cooled to 5°C; 37°C refers to initial dilution of semen at 37°C with EG incorporated in diluter.

[2] Units for treatment comparisons are: No. live = number unstained sperm in 100 counted using a differential live dead stain; motility = units based on an arbitrary 0-5 rating system.

[3] Differences between rating before and after dilution: all differences significant ($.025 > P \geq .001$).

may be that G is more toxic than EG. It has been reported that G is toxic to rabbit sperm in concentrations greater than 5%(2).

In Exp. 4 EG was not significantly different from G in the data based on the proportion of live sperm (11 samples); however, one sample differed markedly in value from all other samples and from its corresponding motility rating, indicating possibly a technical error in staining. Deleting this sample makes the data show EG superior to G ($P<.05$) in proportion of live sperm. Incorporating 8% EG initially at 37°C compared with stepwise addition at 5°C showed significant increase in the proportion of live sperm, corroborating Smith's findings(9). This may be explained by more rapid and easier permeation of the cell at 37°C by EG. The influence of temperature on cell permeability has been demonstrated for red blood cells(14). Two variables confound this comparison of the effects of 37°C *vs* 5° as addition temperatures for EG. They are: (1) equilibration time of 8 hours *vs* 5 hours and (2) all-at-once *vs* stepwise addition of EG. In view of reports on the bovine(17-19) it would seem that the difference in equilibration time would have a negligible effect. Also, the all-at-once addition of EG should, if it has any effect, decrease the amount of pr otection it exerts(5).

Reduction of the concentration of EG or G from 8 to 4% improved both sperm motility and proportion live evaluations. This is in confirmation of Mixner and Saroff's findings(11) on the interference of glycerol with differential staining at levels greater than 4%.

Summary. 1. Ethylene glycol (EG) is significantly superior to glycerol (G) as a diluter constituent for preserving frozen rabbit spermatozoa. 2. The incorporation of EG into the initial diluent, with semen dilution performed at 37°C, is more satisfactory than stepwise addition of EG after semen has been partially diluted and cooled at 5°C. 3. An 8% level of EG or G in the diluter appears to interfere with evaluation of the staining reaction for inviable sperm and it definitely has an adverse effect on motility, as compared with a 4% level.

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