THE TOXICITY OF VARIOUS NON-ELECTROLYTES TO HUMAN SPERMATOZOA AND THEIR PROTECTIVE EFFECTS DURING FREEZING

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Summary. At concentrations varying from 2.5% to 10.0%, glycerol, dimethyl sulphoxide, ethylene glycol, methyl formamide and methyl acetamide had approximately equally toxic effects on human spermatozoa; whereas dimethyl formamide and dimethyl acetamide were considerably more toxic at the same concentrations.

Glycerol and ethylene glycol afforded an equal protective action to human spermatozoa during freezing and thawing, approximately 50% of the original motility being retained, but negligible protection was exhibited by methyl formamide, dimethyl formamide, methyl acetamide and dimethyl acetamide. Only 34% of the original motility was retained when human spermatozoa were frozen in the diluent containing dimethyl sulphoxide.

INTRODUCTION

The fertility of human semen after preservation by freezing has so far proved to be low when compared with that of frozen semen from some other species of mammals (Sadleir, 1966). The only detailed report on the fertility of human frozen semen seems to be by Sawada (1964) who obtained eleven pregnancies from 230 inseminations of 150 women. The low level of fertility of frozen semen may be due to the fact that inseminations are almost invariably carried out on women whose marriages have been infertile, although a contributory factor will be the somewhat reduced motility of the spermatozoa as a result of the freezing process.

The agent most commonly used for the protection of spermatozoa has been glycerol (Sherman, 1963; Freund & Wiederman, 1966) although some work has been done utilizing dimethyl sulphoxide (Zimmerman, Maude & Moldawer, 1964). The effects of other agents, previously described as protecting animal cells during freezing, were investigated and compared with glycerol and dimethyl sulphoxide. They were selected from a group of agents reported by

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Nash (1962) as protecting erythrocytes during the cooling process. Farrant (1964) had also investigated the protective action of these substances on smooth muscle and has reported on their low toxicity when injected into mice.

MATERIAL AND METHODS

Estimation of motility and progression

A thin slide preparation of spermatozoa was made and examined at room temperature (about 20° C). The motility of each preparation was estimated by one of us (D.R.) using: (a) a visual low power estimate to 5% on each of five fields, and (b) a count of all motile and immotile spermatozoa in either a half or a quarter of each of five different fields. The mean of these ten estimates was used as the index of motility of the sample. In addition, the degree of progression of the motile spermatozoa was rated on a scale from 0 (immotile) to 10 (moving so rapidly that tail movement could not be observed).

Protective agents investigated

The following substances were investigated: glycerol, dimethyl sulphoxide, dimethyl acetamide, methyl acetamide, dimethyl formamide, methyl formamide and ethylene glycol.

Toxicity testing

Semen ejaculates obtained from medical students were allowed to liquefy at room temperature and their original motilities determined. Specimens having original motilities below 55% were discarded. Semen from a group of five donors was tested against four concentrations of each protective agent, but the donors in the group were not the same five individuals for each of the seven agents.

The semen from each individual donor was divided into four aliquots which were then each slowly diluted 1:1 with 0.9% saline containing twice the required final concentration of the various protective agents (5, 10, 15 and 20%) and left at room temperature until rated. Motilities and progression ratings of the spermatozoa were estimated at 30 and 60 min after the initial dilution.

Evaluation of protective action

To test the protective effect of the various agents during freezing, concentrations were selected on the basis of the toxicity tests. Fresh liquefied semen specimens from each of another group of six donors were split into aliquots which were then diluted 1:5 with solutions of the semen protective agents in 0.9% saline. In a second experiment, further specimens from the same donors were diluted in the same manner with solutions of the protective agents constituted in Norman-Johnson-1 solution (Freund & Wiederman, 1966). Egg yolk was included in all cases to a final concentration of 15%.

After slow dilution, the semen was equilibrated for 30 min at room temperature, pipetted in 0.5-ml quantities into glass ampoules which were then sealed and cooled at 5° C/min to 4° C and then 1° C/min to -40° C. The ampoules were then stored in liquid nitrogen. They were thawed 39 to 42 days later in a

water-bath at 30° C and removed when the last ice crystal melted. The motility of the sample was estimated 15 min later at room temperature.

RESULTS

Toxicity tests

The average initial motility of the semen samples was 61.3% (SE = 0.80%). The effect of the various treatments is expressed as the percentage loss in motility. Table 1 gives the mean loss in motility after 30 and 60 min for each agent at four different concentrations and the significance of the difference between these means. Examination of Table 1 shows that the different substances had markedly different effects on the motility of spermatozoa although

TABLE 1
MEAN PERCENTAGE LOSS OF MOTILITY IN DIFFERENT CONCENTRATIONS OF PROTECTIVE AGENTS

Agent	Concentration						
	2.5%	P<	5.0%	P<	7.5%	P<	10.0%
<i>30 minutes after dilution</i>							
Glycerol	9.2±2.5	—	16.4±3.8	—	21.2±3.6	0.01	34.6±3.9
Dimethyl sulphoxide	8.4±1.2	0.001	15.2±1.0	0.01	18.2±0.8	0.001	33.6±2.0
Dimethyl acetamide	9.6±5.1	—	19.6±3.7	0.001	36.4±3.8	0.001	76.2±1.6
Dimethyl formamide	11.0±4.5	0.01	23.0±2.3	0.01	43.4±7.6	0.01	78.0±7.8
Methyl acetamide	9.0±2.8	—	13.0±0.7	0.05	18.0±2.9	0.001	33.4±3.5
Methyl formamide	7.4±3.4	—	11.6±2.2	0.05	17.2±1.6	0.001	33.0±2.4
Ethylene glycol	8.0±2.2	0.01	14.2±0.8	0.01	18.8±1.5	0.001	28.8±1.2
<i>60 minutes after dilution</i>							
Glycerol	18.0±2.9	—	25.0±3.7	—	27.6±2.5	0.01	42.6±5.0
Dimethyl sulphoxide	15.8±2.6	0.05	21.6±1.0	0.001	27.0±1.4	0.001	48.0±3.5
Dimethyl acetamide	10.2±2.9	0.001	24.6±1.8	0.001	48.2±3.9	0.001	86.2±1.9
Dimethyl formamide	17.4±4.7	0.01	31.4±4.0	0.01	61.6±9.3	0.01	88.4±5.2
Methyl acetamide	14.0±1.4	0.05	19.0±1.8	0.001	29.8±2.4	0.001	43.4±3.3
Methyl formamide	20.0±3.4	—	24.2±3.3	—	30.8±2.4	0.001	48.8±4.1
Ethylene glycol	14.8±2.0	0.05	18.8±0.6	0.05	23.8±2.2	0.01	34.4±2.8

Mean of five determinations±standard error. The intermediate columns show the degree of significance between the means in adjacent columns.

the second 30 min only reflected differences already shown in the first 30-min period. With the exception of dimethyl formamide and dimethyl acetamide, there appeared to be no consistent pattern in the degree of decrease in motility with increasing concentration of protective agent. However, in the case of these two substances, there was a fairly regular doubling in the loss of motility for each 2.5% increase in their concentrations. The mean progression ratings showed that dimethyl sulphoxide, methyl acetamide and methyl formamide had effects similar to glycerol upon the spermatozoa in concentrations up to 7.5% for 30 min, and 5% for 60 min; but dimethyl formamide and dimethyl acetamide markedly inhibited spermatozoal movement in concentrations above 5% (Table 2).

In high concentrations most of the protective agents had marked inhibitory effects, with one exception, namely ethylene glycol, in which spermatozoa

showed little reduction in progression (except over 10% concentrations) and this characteristic persisted over 60 min. Because of the subjective nature of the progression rating technique, statistical tests were not applied.

Assessment of protective action

The concentrations used in the evaluation of the agents' protective ability during freezing were based upon the results of the toxicity testing; it was decided to use about a 20% loss in motility over 30 min during dilution as the

TABLE 2

MEAN PROGRESSION RATINGS AT DIFFERENT CONCENTRATIONS OF PROTECTIVE AGENTS

Agent	Original rating	30 minutes after dilution				60 minutes after dilution			
		2.5%	5%	7.5%	10%	2.5%	5%	7.5%	10%
Glycerol	8.4	7.8	7.2	6.2	5.4	7.0	6.4	6.2	4.4
Dimethyl sulphoxide	8.0	7.6	7.4	6.8	4.8	6.6	6.4	6.2	3.6
Dimethyl acetamide	8.2	7.8	6.6	4.4	2.0	7.4	5.6	3.8	1.4
Dimethyl formamide	8.2	8.0	7.0	4.8	2.0	7.2	5.8	3.0	1.2
Methyl acetamide	8.8	7.6	7.8	6.2	4.2	6.8	6.6	5.4	3.2
Methyl formamide	8.0	7.6	6.8	6.2	4.0	6.0	5.4	5.1	2.0
Ethylene glycol	8.8	8.8	8.8	7.4	6.2	7.6	7.2	7.0	5.6

TABLE 3

MOTILITY AND PROGRESSION RATING OF SPERMATOZOA AFTER FREEZING AND STORAGE FOR 6 WEEKS (MEAN FOR SIX DONORS)

Agent	0.9% Saline		Norman-Johnson-1 solution	
	Motility (± 1 S.E.)	Progression	Motility (± 1 S.E.)	Progression
Original	61.0 $\pm$ 2.3%	7.8	61.0 $\pm$ 3.1%	7.5
7.5% glycerol	30.8 $\pm$ 1.8%	5.2	34.2 $\pm$ 3.5%	5.8
7.5% dimethyl sulphoxide	23.2 $\pm$ 2.2%	4.7	25.8 $\pm$ 2.5%	3.8
5.0% dimethyl acetamide	<5%	—	<5%	—
5.0% dimethyl formamide	<5%	—	<5%	—
7.5% methyl acetamide	<5%	—	<5%	—
7.5% methyl formamide	<5%	—	<5%	—
7.5% ethylene glycol	29.5 $\pm$ 2.4%	5.5	32.7 $\pm$ 3.5%	5.2

maximum decrease practicable, to enable the extra loss incurred during freezing to be investigated. On this basis, the following concentrations of the agents were used to evaluate their protective ability during freezing: glycerol, dimethyl sulphoxide, methyl acetamide, methyl formamide and ethylene glycol at concentrations of 7.5%; dimethyl acetamide and dimethyl formamide at concentrations of 5%.

Protective action during freezing

Dimethyl acetamide, dimethyl formamide, methyl acetamide and methyl formamide had a negligible protective action, the spermatozoa, after freezing

and thawing in solutions of these substances, had levels of motility below the minimum assessable by the methods used (Table 3).

The motilities, after thawing, of spermatozoa frozen in saline solutions of glycerol or ethylene glycol were very similar, but the motility was significantly lower with dimethyl sulphoxide when compared statistically to glycerol ($P<0.01$) and ethylene glycol ($P<0.05$). The general motility after thawing was slightly, but not significantly, higher when Norman-Johnson-I solution was used. In this medium, the post-thaw motility of spermatozoa in dimethyl sulphoxide was significantly lower than in glycerol ($P<0.05$) but not significantly lower than in ethylene glycol. After freezing, storage and thawing, the progression rating had dropped in all three substances. Again, ethylene glycol had a protective action similar to that of glycerol, but dimethyl sulphoxide was more inhibiting to the progression of spermatozoa.

DISCUSSION

Dimethyl sulphoxide and glycerol are the only substances of the group tested in this study, whose effect on human spermatozoa has been previously reported (Zimmerman *et al.*, 1964). After freezing, storage and thawing, these workers reported loss of motilities of 62% for 10.0% glycerol, 75% for 10.0% dimethyl sulphoxide and 81% for 5.3% dimethyl sulphoxide. In the present study the losses of motility were 44 to 49% for 7.5% glycerol and 58 to 62% for 7.5% dimethyl sulphoxide, so that it would appear that this more successful recovery may be due to 7.5% being nearer to the optimal concentration of these substances required for freezing. Sawada (1964) found that concentrations of glycerol higher than 9.1% depressed the motility of spermatozoa before freezing, and considered that 7.7% was the optimal concentration of glycerol for preservation. Zimmerman *et al.* (1964) maintained that the higher the motility of the spermatozoa in the sample before freezing, the better the survival of spermatozoa after freezing. No evidence of such a correlation was found in this study.

Emmens & Blackshaw (1950) found that 7.5% ethylene glycol protected rabbit spermatozoa somewhat during freezing. They also investigated the properties of human seminal plasma but did not report on the effect of ethylene glycol on human whole semen. The data reported here indicate that the protective action of ethylene glycol is similar to that of glycerol which suggests that the fertility of human semen frozen in these agents should be compared. Jones (1965) has reported that the best recovery rates of ram semen were obtained after freezing in mixed solutions of 7% glycerol and 1.5% dimethyl sulphoxide in combination. It is possible that this approach will result in higher recovery rates.

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