Studies on the motility and cryopreservation of rainbow trout (*Salmo gairdneri*) spermatozoa

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The very short duration of vigorous movement (1½ to 7 min) in fresh water and physiological solutions make trout spermatozoa difficult subjects for cryopreservation studies. Solutions consisting of 250 to 280 mmol sucrose and 5 to 12% dimethyl sulphoxide (DMSO) (4 parts) did not activate trout spermatozoa (1 part), but after dilution with fresh water vigorous motility could be fully restored. These sucrose-DMSO solutions were employed in cryopreservation studies. Using straws and a fast freezing — fast thawing procedure, post-thaw dilution with fresh water resulted in 25% – 60% of spermatozoa becoming motile, all with vigorous forward progression. Some existing methods for the cryopreservation of other freshwater fish spermatozoa were repeated on trout without success.

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Die baie kort tydsduur van aktiewe beweging (1½ tot 7 min) in vars water en fisiologiese media, bemoeilik die bevriesing van forelspermatosoë. Oplossings bestaande uit 250 tot 280 mmol sukrose en 5 tot 12% dimetielsulfoksied (4 dele) aktiveer nie forelspermatosoë (1 deel) nie, maar na verdunning met vars water herstel aktiewe beweging van spermatosoë heeltemal. Hierdie sukrose-DMSO oplossings is gebruik vir die bevriesingstudies. Wanneer strooitjies en 'n vinnige vries — vinnige ontdooiing prosedure gebruik is, het verdunning met varswater na ontdooiing veroorsaak dat 25% – 60% van die spermatosoë beweeglik geraak het en almal het kragtige voorwaartse beweging getoon. Sommige van die bestaande metodes vir die diepvriesbewaring van ander varswatervis-spermatosoë is sonder sukses herhaal.

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Although several attempts have been made during the past decade to cryopreserve trout spermatozoa, fertilization rates were either low or variable. Graybill and Horton (1969) obtained a fertilization rate of 18% using a saline-fructose-lecithin based extender, Ott and Horton (1971) were more successful with a bicarbonate-mannitol-lecithin extender (59%), Büyükhatipoglu and Holtz (1978) could not repeat this latter work but used yet another extender (based upon trout seminal plasma) and obtained 2% – 80% fertilization. All of these extenders included 12% dimethyl sulphoxide (DMSO).

When freshwater fish spermatozoa are collected without exposing them to water (milt) they are inactive and they can be kept in that state at 4°C for up to 24 h. During this time the spermatozoa will become motile if the milt is diluted with water or a physiological salt solution. Once activated, trout spermatozoa remain vigorously motile for 29 – 60 s (Scheuring 1928, Schlenk & Kahman 1937, Medem, Rötheli & Rath 1949). Either the vigorous motile life span of the sperm may be extended significantly in order to allow time for cryopreservation procedures or an extender that will not activate the spermatozoa can be used during the freezing procedure and motility initiated by further dilution after thawing.

Ginzburg (1972) indicated that a positive correlation exists between fertilization rates and good forward progressive motion in trout spermatozoa. We have therefore used sperm motility as a parameter to test the effect of various extenders and procedures for cryopreservation before testing for fertilizing ability. The latter parameter would in any case also be influenced by the condition of the eggs.

Materials and Methods

Milt was collected from ripe, two to three year old rainbow trout either maintained in tanks at 4°C or kept at a trout hatchery. Before stripping milt the area around the urogenital papilla was dried and pressure was exerted on the bladder thereby forcing urine out in order to avoid possible contamination with the milt.

Milt was collected in precooled (4°C) test tubes and afterwards maintained at 4°C in a cold room. Of these only spermatozoan samples exhibiting Grade 3 motility (see later) and more than 90% motile spermatozoa on dilution with fresh water were used.

Assessment of motility

None of the methods used for quantifying motility of mammalian spermatozoa were suitable for trout spermatozoa because of the long (120 s) equilibration time required after the spermatozoa sample had been put on the slide (Dott & Foster 1979), therefore motility was graded using phase contrast microscopy at a magnification of 200× and 400×. To exclude bias as far as possible, the observer grading the motility was kept unaware of the solutions being tested.

There were four grades of motility; grade 3 — virtually all spermatozoa move vigorously and most spermatozoa swim progressively forwards; grade 2 — although most spermatozoa move vigorously only approximately 50% swim progressively forward; grade 1 — a few to many motile spermatozoa, although virtually no spermatozoa swim progressively forward; grade 0 — all spermatozoa immotile. The approximate percentage of motile spermatozoa was also assessed.

Experiments on duration of spermatozoan motility

A stop watch was started as the milt was thoroughly mixed with each solution to give a final dilution of five times. The initial motility grade and the duration of each motility grade was recorded. This was relatively easy because the entire population of motile spermatozoa appeared to change from one type of motility to another almost simultaneously. However, attention was primarily devoted to the duration of vigorous spermatozoan motility (Grades 3 and 2).

Solutions used for motility studies

Table 1 outlines the concentration and composition of the solutions used for the motility studies on rainbow trout spermatozoa in this investigation. Analytical grade chemicals were used.

Cryopreservation (Extenders and procedure)

Milt was mixed with solutions containing 250 mmol sucrose or 280 mmol sucrose and 5, 8, 10, 12, 14 and 16% DMSO respectively at 4°C. One to five hours was allowed for equilibration. Milt was also mixed 1:5 with solutions containing 250 mmol sucrose and 3,5 and 10% glycerol respectively at 4°C.

The methods of Ott and Horton (1971) for the cryopreservation of trout spermatozoa and that of Mounib (1978) for Atlantic salmon spermatozoa (freshwater spawner) were also repeated for rainbow trout spermatozoa in this investigation.

In preliminary trials with pellet freezing we obtained poor post-thaw motility, probably because the drops were too large to allow sufficiently rapid freezing and/or thawing. After preliminary trials with straw freezing, ministraws (0,25 ml) containing diluted semen were frozen in liquid N_2 vapour for 7 – 9 min and then plunged into liquid N_2. Fast thawing was achieved by putting the frozen straws into a water bath at 27 – 35°C for 4 – 5 s. This provided the fast thaw necessary before dilution with fresh water at 4°C to reactivate the spermatozoa.

Table 1 Concentration and composition of solutions used for trout sperm motility studies

Solution	Composition	Concentration mmol	mOsm/kg	%
Sodium chloride		1,2	30	
		53	100	
		80	150	
		108	200	
		118	220	
		150	276	
		162	300	
Sucrose		90	50	
		125	100	
		150	150	
		180	200	
		195	220	
		250	273	
		280	300	
Dimethyl sulphoxide		70		5 (v/v)
(DMSO)		113		8 (v/v)
		140		10 (v/v)
		169		12 (v/v)
		197		14 (v/v)
		225		16 (v/v)
Glycerol		41		3 (v/v)
		68		5 (v/v)
		136		10 (v/v)
Egg yolk				3 (v/v)
				5 (v/v)
Mounib 1978	sucrose	125		
	reduced glutathione	6,5		
	$KHCO_3$	100		
	DMSO	169		12 (v/v)
	Total concentration		283	
Reduced glutathione		6,5	7	
Potassium bicarbonate		100	152	
Ott and Horton 1971	$NaHCO_3$	8,6		0,72 (w/v)
	lecithin	1,5		0,75 (w/v)
	mannitol	1,4		0,25 (w/v)
	DMSO	169		12 (v/v)
	Total concentration		187	
Glucose		10	9	
Mannitol		100		
Sodium bicarbonate		100		
Potassium chloride		100		
Deionized water			1	
Trout hatchery water			10	
Sucrose		125		
NaCl		100		
	Total concentration		230	

Results

Activation studies

All concentrations of NaCl tested activated spermatozoa, as did concentrations of sucrose below 195 mmol (Table 2). If the milt was diluted at 1:10 instead of 1:5, sucrose above 195 mmol also activated. Distilled water activates trout spermatozoa, so solutions which activate may be thought of as permissive while those which do not can be considered as inhibitory.

If a solution did not inhibit activation, its value was assessed by comparing the durations of the various motility grades in the solution with their durations in water (see Table 3). Vigorous sperm motility lasted longer in some of the solutions than in water but none of them extended the duration of vigorous motility enough to allow pre-freeze manipulation of the diluted semen.

Table 2 Activation of trout spermatozoa by dilution at 4°C. (For each solution n = 5) (Motility Grade, (MG) 3 = + + +, MG 2 = + +, MG 1 = +, MG O = −)

	Osmotic concentration (mOsm/kg)	Sperma-tozoan dilution	Activation and motility grade	Activation after dilution with fresh water and motility grade
NaCl (1,2 to 162 mmol)	30 to 300	5	+ + +	
Sucrose (90 to 180 mmol)	90 to 200	5	+ + +	
Sucrose (195 to 280 mmol)	220 to 300	5	−	+ + +
Sucrose (195 to 280 mmol)	220 to 300	10	+ + +	
DMSO (5—12%)	−	5	+ + +	
DMSO (14—16%)	−	5	−	−
Glycerol (3—5%)	−	5	+ +	+ + +
Glycerol 10%	−	5	+	+
Egg yolk 5—10%	−	5	+	+
Mounib 1978	283	5	−	−
reduced glutathione 6,5mmol	7	5	−	−
$KHCO_3$ (100 mmol)	152	5	−	−
(Ott and Horton 1971)	187	5	+ + +	
glucose (10 mmol)	9	5	−	−
mannitol (100 mmol)	−	5	+ + +	
lecithin (1,5 mmol)	−	5	+ + +	
$NaHCO_3$ (100 mmol)	−	5	+ + +	
KCL (100 mmol)	−	5	−	−

On the other hand some solutions which inhibited activation later permitted it when further diluted with fresh water. Sucrose at 250 mmol did not activate when used as an extender at a dilution rate of 1:5 but the spermatozoa became vigorously motile when diluted further with fresh water (Table 2). The extender of Ott and Horton (1971) activated trout spermatozoa immediately whereas that of Mounib (1978) did not, even when further diluted with fresh water.

Cryopreservation studies

The results of the cryopreservation studies are summarized in Table 4. Sucrose (250 mmol) was used as the extender for most of these experiments with various concentrations of DMSO or glycerol as cryoprotectants. Post-thaw recovery was appreciable using DMSO. All levels of DMSO resulted in about 35% of spermatozoa becoming motile after the thawed extended semen was diluted with fresh water, and these spermatozoa exhibited grade 3 motility. On the other hand, glycerol proved a much less effective cryoprotectant; only about 3% of the spermatozoa became motile and these only showed grade 1 motility. When 280 mmol sucrose was compared to 250 mmol sucrose, in combination with DMSO, the higher concentration did not improve post-thaw recovery. No recovery at all was obtained when the DMSO containing extenders of Ott and Horton (1971), or Mounib (1978) were used.

Discussion

Trout spermatozoa in milt remain inactive at 4°C for at least 24 h (unpublished observations). The spermatozoa become vigorously motile when fresh water at 4°C is added at any time during this period, but the vigorous motility persists for less than 1 min. We have observed that if dilution of the milt is 1:5 the presence of NaCl, $NaHCO_3$, mannitol, lecithin, or up to 12% DMSO, does not prevent activation; on the other hand, KCl, $KHCO_3$, reduced glutathione, 14 – 16% DMSO, or glucose inhibit activation, and only feeble motility is obtained in the presence of glycerol or egg yolk (Table 2).

At a dilution rate of 1:5, up to 190 mmol sucrose permitted activation, whereas concentrations greater than 195 mmol inhibited it. On the other hand, if the milt was diluted 1:10, the higher concentrations of sucrose were no longer inhibitory. In view of this it was not surprising to find that the inhibition of motility by high concentrations of sucrose could be overcome by further dilution with fresh water. Inhibition by glycerol was also reversed by further dilution with fresh water, whereas spermatozoa in KCl, $KHCO_3$ or glucose remained inactive after further dilution. One explanation of these results is that there are 2 types of inhibition, an 'active' inhibition exhibited by K^+ or glucose, and a 'passive' inhibition by sucrose; the latter might result from an endogenous inhibitor in or on the trout spermatozoon which is soluble in water but only slightly soluble in a sucrose solution.

Vigorous motility following activation of trout spermatozoa is short-lived yet it is apparently a pre-requisite for good fertilizing ability Ginzburg (1972). It seems logical therefore to carry out cryopreservation either in an extender that prolongs vigorous motility or in an extender that inhibits activation in a reversible fashion.

Table 3 The duration of trout spermatozoon motility in various solutions (spermatozoa diluted × 5) at 4°C

		Duration of vigorous motility (s)				
		Grade 3		Grade 2		
Sperm diluent	Osmotic concentration mOsm/kg	Average $n = 2$	Range	Average $n = 2$	Range	Total duration of motility (s)
Deionized water	–	90	82—98	24	20—28	457
Fresh water	10	86	81—90	27	21—33	428
Sucrose	50	111	107—115	42	35—43	309
,,	100	109	108—110	26	20—32	340
,,	150	110	105—115	37	29—45	280
,,	200	123	120—126	17	10—24	245
,,	220	0	—	—	—	0
,,	250	0	—	—	—	0
Sodium chloride	30	75	74—78	35	30—40	157
,,	100	84	80—88	117	105—130	350
,,	150	104	91—118	91	60—121	370
,,	200	143	121—165	104	74—134	>600
,,	220	144	133—155	118	69—167	>600
,,	276	165	159—172	209	202—216	>600
,,	300	157	137—178	117	74 – 160	>600
Sucrose : NaCl (1:1)	230	122	118—126	116	105—127	>600

Huxley (1930) found that the motile life of trout spermatozoa could be prolonged in saline solutions. Our results confirmed this (Table 3, final column) but the duration of vigorous motility could be increased only to approximately 6 min, not long enough for freezing to be attempted. Sucrose at concentrations greater than 195 mmol inhibited activation reversibly and in combination with DMSO enabled trout spermatozoa to be frozen and thawed with a recovery of motility sufficiently high (25 – 60%) to suggest a high fertilization rate. Similar recovery rates were reported by Mounib (1978) with frozen Atlantic salmon spermatozoa, when an 85% fertilization rate was obtained.

The cryopreservation procedure reported by Büyükhatipoglu and Holtz (1978) for trout spermatozoa which yielded variable results (2 – 80% fertilization) but seems to be the most successful method so far reported, appeared too late to be tested.

Although our method can not yet be regarded as successful, it appears from this investigation and the literature that future studies should involve more detailed research of basic trout spermatozoon physiology. Special attention should be devoted to activating and inhibiting substances on spermatozoon motility. It was for example shown that the monosaccharides glucose and mannitol have opposite effects on trout spermatozoon motility (Table 2). The former renders spermatozoa immotile while mannitol as used in the extender of Ott and Horton (1971) can induce vigorous spermatozoon motility. The underlying principles of phenomena like these should be investigated as well as the quantification of trout spermatozoon motility in relation to fertilization in order to have a better scientific basis and rationale for developing cryopreservatives in future studies.

Table 4 Cryopreservatives used and spermatozoan motility results after thawing and dilution with fresh water ($n = 5$ for each cryopreservative) (% v/v)

		Percentage motile spermatozoa	
Cryopreservative	Motility grade after thawing and dilution	Average	Range
250 mmol sucrose, 5% DMSO	3	40	30—60
,, , 8% DMSO	3	35	25—55
,, , 10% DMSO	3	35	30—55
,, , 12% DMSO	3	35	30—50
280 mmol sucrose, 5% DMSO	3	35	30—55
,, , 8% DMSO	3	40	30—60
,, , 10% DMSO	3	30	25—55
,, , 12% DMSO	3	35	30—55
250 mmol sucrose, 3 to 10% glycerol	1	3	0—5
Mounib 1978	0	0	
Ott and Horton 1971	0	0	

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