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Effect of Equilibration Times on Sperm Cryopresevation of Juvenile Rainbow trout (*Oncorhynchus mykiss* W., 1792)

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Abstract

The effects of four equilibration times (0, 5, 10 and 15 min) and three diluents (0.3 M glucose, 0.6 M sucrose and Erdahl and Graham's) on post-thaw spermatozoa in juvenile Rainbow trout (*Oncorhynchus mykiss* W., 1792) were examined by sperm motility percentage and duration. The sperm was frozen in straws in liquid nitrogen vapour and stored for one weeks in liquid nitrogen (LN2). They were then thawed in a 25 º C water bath for 30 s. Among the four different equilibration times tested, 0 and 5 min yielded the highest mean post-thaw motility percentage ($P<0.001$). The 10 and 15 min equilibration of spermatozoa in diluents before freezing significantly lowered the duration of post-taw sperm motility ($P<0.001$). No significant difference among three diluents was observed in post-thaw motility percentage and duration at each equilibration times. Our results showed that any equilibration time between 0- and 5-min in 0.3 M glucose, 0.6 M sucrose or Erdahl and Graham's diluent was suitable equilibration time for R.trout sperm.

Key words: Cryopreservation, diluent, equilibration time, motility, *Oncorhynchus mykiss*.

INTRODUCTION

Collection and cryopreservation of good quality sperm for future use may employe the fit of artificial insemination and reduce stress to male kept for breeding caused by repeated sperm sampling which reduces sperm quality [1]. Sperm cryopreservation has been used in hatcheries to facilitate artificial reproduction of fishes and genetic improvement, minimize the cost of maintaining broodstock and reduce the risk of disease transmission [2]. Also cryopreservation permits long-term storage of sperm for use when quality has declined and synchronized male and female gamete availability for strip spawning [3–6]. Access to frozen semen creates likely to use specific male fishes regardless of the time or place. Furthermore, the long-term sperm storage is required to maintain genetic resources.

There are several damaging factors when spermatozoon is frozen: thermal shock, formation of ice crystals, dehydration, increased salt concentration and osmotic shock [1, 7–10]. The freezing and thawing of fish sperm involves some loss of spermatozoon's motility [8, 9, 11–14].

Over the years more complex extenders have been introduced in an attempt to improve the recovery of functional spermatozoa after freezing. Many studies have evaluated various extender including ions, hepes, bovine serum albumin, egg yolk, sucrose, glucose, citrate [8, 12, 15–17]. Optimum cryosurvival is dependent also on the cryopreservative material used. Glucose, sucrose and Erdahl and Graham' diluent, which contains DMSO, (Dimethyl Sulfoxide DMA (Dimethyl acetamide) , EG (Ethyleneglycol), Glycerol, Propanediol, Methanol, PVP (Polvinyl pyrr as cryoprotective agents were used as extenders in fish species [5, 8]. In addition, Robles et al. [17] found that the diluent consisting of 10 % DMSO and 90 % 300 mM glucose solution had a low percent motility in rainbow trout.

Many kinds of diluents and different combinations among the components are used for preservation of R.trout sperm [8, 15–17]. However, the freezing procedure reduces motility and duration of spermatozoa. Thus, many frozen sperm could not use for artificial insemination. It is a commonly known fact that the spermatozoa are exposed to the cryoprotectant prior to freezing has effects on its post-thaw characteristics. A significantly increased post-thaw motility as well as fertility for an equilibration time of 2 min for sea cucumber Apostichopus japonicus (Selenka) sperm, compared to 8- and 15-min equilibration times has been described [10]. In a previous study, it was only suggested that equilibration of rainbow trout spermatozoa before freezing is not needed [5].

The aim of this study was to determine the effect of different equilibration times with glucose, sucrose and Erdahl and Graham' diluent on post-thaw spermatozoa motility and duration in rainbow trout milt.

MATERIAL AND METHODS

Animals

Sperm were obtained in March 2005 from juvenile 1 year old male rainbow trout spawners cultured in the Department of Fisheries, Harran University Bozova Vocational High School,

Bozova, Sanliurfa, TURKEY. The size of fish (means ± S.E.) used for the study was 246.8 ±17.4 g in body weight and 14.08± 0.4 cm in total lenght.

Collections of sperm

Sperm were collected at near the fish cages by abdominal massage and kept on ice for no longer than 3 hours. The motility percentage was determined visually by estimating the proportion of motile and non-motile cells after 1: 500 dilution with the activation solution. The duration of sperm motility was evaluated as the time elapsed from activation until 5 % of the spermatozoa maintained forward swimming activity. An activation solution, 50 mM NaCl (20 mM Tris-HCl, pH 8.0), was used for estimating motility rate. Spermatozoa that vibrated in place were not considered to be motile. The duration of progressive movement was also recorded as expressed second [18].

The percentage of motile spermatozoa was considered decesive factor. Semen samples with at least 80 % progressive motility were pooled. The sperm was kept at 4 ºC until freezing.

Experimental Design

Different equilibration times were tested depending on the diluent. Three diluents were tested in combination with the effect of equilibration time. The next three diluents were chosen: organic, 0.3 M glucose and 0.6 M sucrose [6] and mineral, seminal plasma-mimicking, Erdahl and Graham's [5]. The latter consisted of: 0.29 g CaCl2.H20; 0.4 g MgC12.6H20; 0.5 g Na2HPO4; 5.1 g KCI; 11.7 g NaC1; 0.2 g citric acid; 20 g glucose; 20 ml of 1.27 g/100 mL KOH; 20 ml of 5.3/100 ml bicine; supplemented to 2 L with distilled water.

Sperm freezing

Semen samples from six males were pooled in equal amounts and diluted with 0.3 M glucose, Erdhal and Graham's or 0.6 M sucrose solution containing 10% DMSO and 10 % egg yolk at a 1:3 (sperm: extender) ratio at 4 º C. Diluted sperm was transferred into 250 µl straws tubes (IMV, France), which were then placed into refrigerator (4 º C) for equilibration (time between sperm dilution with the extender and the start of freezing). They were equilibrated for 0, 5, 10 and 15 min. The straws were frozen at 5 cm above the liquid nitrogen surface in a closed styrofoam box for 10 min and were then plunged into the liquid nitrogen. Straws were stored in a liquid nitrogen tank until the motility assessment was performed, one week later.

The experiments were carried out in triplicate using three different pools of semen.

Spermatozoa motility and duration assessment

Frozen sperm were thawed in a 25 º C water bath for 30 s after one week of storage. Motility and duration of thawed semen were estimated under the the binocular light microscope (at 400x magnification) at least five times in each treatment. In cryopreserved semen, motility and duration analysis were performed immediately after thawing as previously described.

Statistical Analyses

Spermatozoa motility and duration obtained from each diluent and equilibration time were expressed as percentage (means ± SE) and s, respectively. Motility data were normalized through arcsine transformation and the results were analyzed using a multifactor analysis of variance (MANOVA). Significant differences between treatments were detected using the Duncan's multiple range test ($P < 0.05$) generated by SPSS software (SPSS 10.0 windows).

RESULT

In motility percentage, an interaction was found between diluents and equilibration time ($P<0.001$). Because an interaction was found, a nonfactorial ANOVA with a Duncan's multiple range test was performed on this two traits.

Equilibration time had a significant effect on the % motility of spermatozoa (Fig. 1). The % motility of post-thaw spermatozoa was not siginificantly different when equilibration time was performed at 10 or 15 min but increased ($P<0.001$) when equilibrated at 0 or 5 min.

When the effect of diluents was tested, the best results (54.58 %) were achieved using 0.6 M sucrose and 0.3 M glucose diluent (Fig.2). The lowest mean % of post-thaw motility was observed in Erdahl and Graham's diluent (Fig.1).

The mean duration of post-thaw motility was siginificantly ($P<0.001$) higher at 0- and 5- min equilibration time compared to the other two equilibration times. The lowest duration of post-thaw motility was observed at 15 min equilibration time (Fig 3).

Spermatozoa frozen in 0.6 M sucrose diluent had the highest duration of post-thaw motility. However, this parameter was not siginificantly different ($P>0.05$) from values obtained in the other diluents tested (Fig. 4)

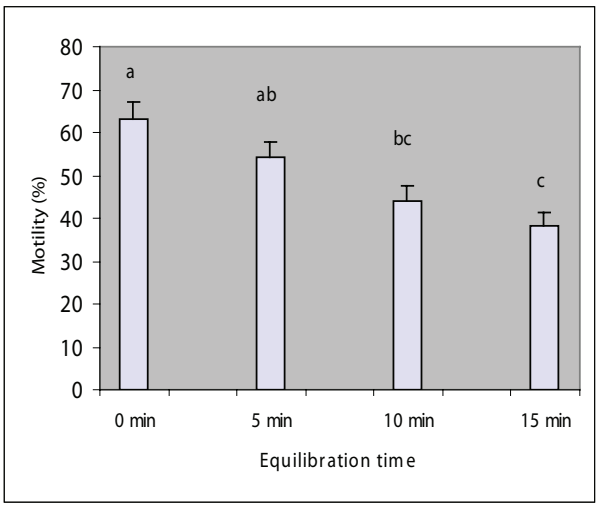

Figure 1. Effect of different equilibration periods on post-thaw motility percentages in R. trout sperm. "a,b,c" : different letters are significantly different at $P < 0.05$. Results for each equilibration period represent the mean (± SE) of the three cryopreservation diluent. Sperm from six males were pooled prior to cryopreservation.

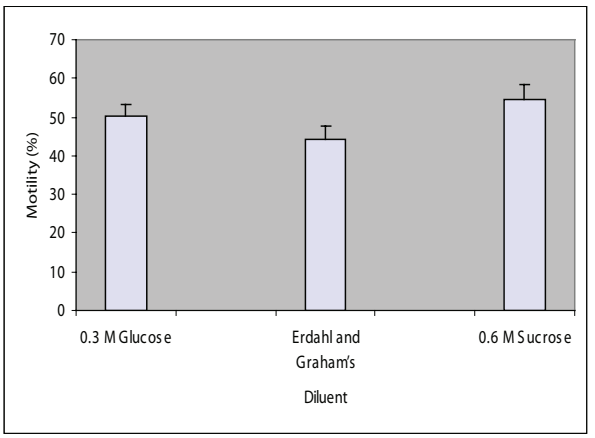

Figure 2. Effect of different diluent on post-thaw motility percentages in rainbow trout sperm. There were no significant differences among the three diluents. Each point represents the mean ± SE of samples from ten fish pooled prior to cryopreservation.

DISCUSSION

The results of this study demonstrated that the equilibration time had a siginificant effect (P<0.001) on the post-thaw % motility and duration of motility of juvenile R. trouts. The best motility % of spermatozoa was obtained when straws were equilibrated at 0 and 5 min. Babiak et al. [5] reported that the equilibration of sperm in diluent before freezing had a generally harmful effect in R. trout. Stoss and Refestie [19] suggested that DMSO had a toxic affect during equilibration time when the time was extended which was similar to our result. On the contrary, in another R. trout study in which 15 min equilibration time was applied to extended milt, Robles et al. [17] notified no loss in motility. Although they were not compare the effect of equilibration time on sperm motility, that study suggested that differences in freezability could be probably due to differences in plasma membrane composition [17]. In the present study, too long durations of equilibration time were not found suitable for cryopreservation. Perhaps the conflicting findings are due to variations in cryoprotectant concentration used. Also, Billard et al. [20] suggested that the equilibration time should be kept to a minimum mainly to minimize the cryoprotectant toxicity. Thus, it is likely that there are differences in sperm membrane composition between the two population of the fishes.

Both mineral and saccharides have been successfully used in diluents for sperm of R. trout [5, 8, 14, 17]. In the present study, although semen diluted with simple carbohydrate-based diluents (0.6 M sucrose and 0.3 M glucose diluent) demonstrated high post-thaw motility % compared to sperm diluted with mineral-based diluent (Erdahl and Graham's diluent) in the R. trout but did not changed siginificantly when diluted in three dilutent. Accordingly, Lanes et al. [21] reported better motility after freezing Brazilian flounder (Paralichthys orbignyanus) sperm using DMSO-sucrose based extender (2.5±0.3) in comparison with glycerol–saline based extender (1.3±0.4). In addition, an interaction between diluents and equilibration time (P<0.001) was found, it could be explained by the differences in the usefulness of diluent constituents for R. trout sperm [5]. The presence of fresh hen egg yolk in the freezing dilunet is usually considered to protect the cell membranes during the freezing processes [22]. Contrary to our study, it had been found to be harmful when using 0.3 M glucose. Babiak et al. [5] reported that effect of 10 % addition of egg yolk to the extender on the fertilizing ability of cryopreserved spermatozoa was strongly dependent on the type of diluent used.

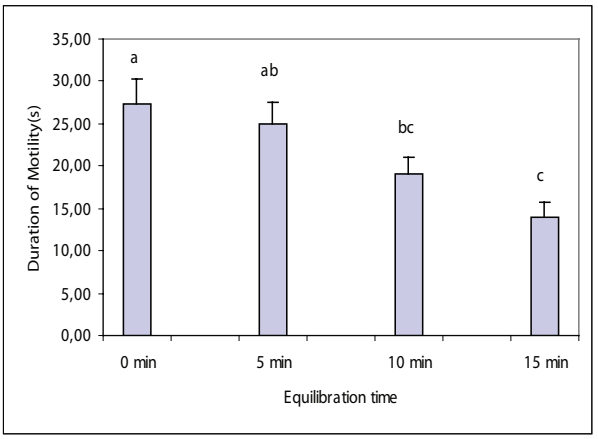

Figure 3. Effect of different equilibration periods on duration of post-thaw motility in rainbow trout sperm. "a,b,c" : different letters are significantly different at P < 0.05.Equilibrium times are for three cryopreservation diluents and that the values are the means (± SE) of data obtained from all three diluents.

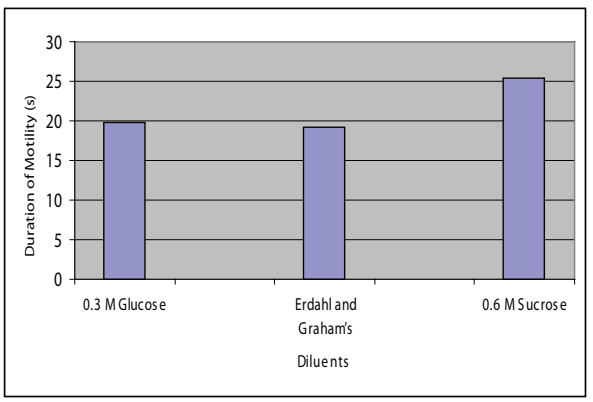

Figure 4. Effect of different diluent on duration of post-thaw motility (mean± SE) in rainbow trout sperm. There were no siginificant differences among the three diluents. Each point represents the the mean ± SE of sperm from ten fish pooled prior to cryopreservation.

Although several mechanism have been recommend to the exact manner of activity, including that for frozen R. trout sperm, remains to be elucidated.

Previous studies of salmonidae have demonstrated that freezing in cryodiluent containing cryoprotectant causes visible motility loss [8, 11, 16]. However, the results of the present study indicate that sperm equilibrated at 10 or 15 min was linked with a greater loss of duration of post-thaw motility than was equilibrated at 0 and 5 min. The investigators [7, 8, 9, 16] indicated that freezing components appear to be important factors influencing membrane stability and motility, largely because high cryoprotectant concentration perform severe osmotic effect. Diluents had no significant effect on duration of post-thaw sperm motility for R. trout. Duration of post-thaw motility in frozen semen was observed to be low compared to that of fresh sperm. The findings of this study was supported by Lahnsteiner et al. [11] who reported that freezing process decreases the duration of motility in cryopreserved semen.Our results suggested that motility loss can be unlinked osmotic effect, because we

added the classic concentration of DMSO. Obviously, in the present study, prolonged exposure to conventional DMSO concentration had harmful effect to spermatozoa function.

Because a high rate of progressive motile spermatozoa is one of the prerequisites for fertilization it can be concluded that any equilibration time between 0 and 5 min is suitable for freezing sperm from R. trout when either 0.3 M glucose, 0.6 M sucrose or Erdahl and Graham's diluent is used. In addition, all diluent used had a similar effect on sperm freezing in R. trout. Results obtained in this study may be valuable for developing the freezing process for cryopreservation of R. trout sperm. Further advances in freezing process are required to improve the utilization of frozen sperm.

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