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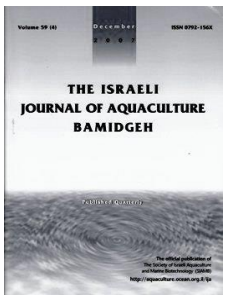

The Effect of Streptomycin on Freezing Rainbow Trout (*Oncorhynchus mykiss*) Sperm

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Abstract

Mature male and female rainbow trout (*Oncorhynchus mykiss*) were used to study the effect of different concentrations of streptomycin in a cryopreservation solution on the sperm motility, motility duration, and fertilization rate of rainbow trout. 300mM glucose and 10% DMSO containing diluent was used as a cryopreservation solution. Three different concentrations (3600, 7200 and 10800 µg/ml) of streptomycin were added to the diluent. Our study showed that various concentrations of streptomycin in cryopreservation solution caused a significant reduction in the percentage and duration of rainbow trout spermatozoa motility after freezing-thawing. The percentage of egg fertilization also decreased. When streptomycin-containing groups were compared with each other, a statistical difference was found ($p<0.05$). However, no statistical difference was observed in terms of the percentage of eyed-embryos.

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Introduction

In routine or special-purpose fertilization studies sperm cryopreservation is used for the purpose of long-term sperm preservation of extinct or endangered species (Purdom, 1993). It is a safe and effective method for the protection of genetic resources, and is also used to protect new lines, and specific features of endangered species (FAO/UNEP, 2000). The first successful cryopreservation studies on fish sperm were carried out by Blaxter in 1953 using the sperm of herring (Purdom, 1993). Since then studies on semen cryopreservation of a large number of fish species (salmonids, Cyprinidae, turbot, etc.) have been carried out using different diluents (Suquet et al., 1995; Tekin et al., 2003; Bozkurt and Seçer, 2005; Li et al., 2010). In order to ensure high survival of thawed spermatozoa, cryoprotective ingredients such as sugar, egg yolk, and a variety of chemicals are used (Purdom, 1993; Salvetti et al., 2006). Many extenders containing different cryoprotective media are used; glucose and DMSO are commonly used with successful results (Bozkurt et al., 2005; Tekin et al., 2007; Tekin et al., 2003; Ekici at al., 2012). Rainbow trout have been widely used in cryopreservation applications.

Antibiotics are used for the elimination of sexually transmitted diseases (Salvetti et al., 2006). It has been reported that penicillin and streptomycin are also used to prevent contamination during the preservation of the sperm of farm animals (Salvetti et al., 2006). Antibiotics are biologically active substances which are thought to affect cell function (Magli et al., 1996). Streptomycin, one of the antibiotics used in laboratory studies, is used in mammals for sperm washing, and in cryopreservation media to control fungal and bacterial growth (Magli et al., 1996; Khaki et al., 2008).

The most commonly used antibiotics are streptomycin sulfate and penicillin (Magli et al., 1996) and they are often used in combination (Khaki et al., 2008). The addition of penicillin and streptomycin to the culture medium is reported to be quite effective in reducing the frequency of microbial contamination observed in the sperm wash and swim-up period (Magli et al., 1996). During short-term storage of some types of fish sperm at 4-6°C, various antibiotics are used to prevent contamination.

Some studies have shown that antibiotics had positive effects on sperm motility (Saad et al., 1988), while in other studies these positive effects were not observed (Maria et al., 2006). Doses of between 500-6000 IU of penicillin and 500-6000 µg/ml streptomycin were used to prevent bacterial and fungal contamination during short-term preservation of rainbow trout sperm (Stoss et al., 1987; Stoss, et al, 1978). Sperm may contain pathogens (Russell et al., 1997), so the risk of sperm contamination may occur during short-term preservation (Ciereszko, 2007). In our study, we used streptomycin to prevent the risk of sperm contamination during the experiment.

Materials and Methods

Fish Care and Experimental Design. This study was carried out at Istanbul University, Faculty of Fisheries, Sapanca Inland Waters Research Center. The stripping process was carried out on 2-3 year old male, and 3-4 year old female, rainbow trout (*Oncorhynchus mykiss*) held at 10±1°C water temperature. The equipment, solutions, and semen samples obtained through the abdominal massage stripping method and stored in 50 ml sterile glass beakers, were kept at 4ºC during the experiment.

Spermatozoa motility observations. 19µl of activation solution (0.3% NaCl) was added to 1µl semen samples and spermatozoa motility was examined under a light microscope with a magnification of 40X. Spermatozoa samples with motility over 85% were used in the trials. Spermatozoa motility observations were made using three replicates per sample. Motility was determined using a 5 point scale method, where 5 = rapid forward motion of all spermatozoa (Morisawa, et al., 1986; Dettlaff, et al., 1993; Cosson, 2007). A Hayem solution (5g Na_2SO_4, 1g NaCl, 0.5g $HgCl_2$, and 200ml bidistilled water) together with the hemocytometric method were used to determine spermatozoa density.

Cryopreservation and Storage. A cryopreservation solution of 300mM glucose (Merck) and 10% DMSO (Sigma) was used. This diluent, which did not contain streptomycin (Sigma), was the control. Three different concentrations of streptomycin (3600, 7200, and 10800 µg/ml) were added to this diluent in the experimental groups. A sperma pool was created from semen with motility over 85%, taken from three male fish. Before cryopreservation, the pooled sperm samples were diluted at a 1:2 ratio (sperm/extender) using a cryopreservation solution. Diluted samples were immediately drawn into 0.25ml straws. In the study of sperm freezing in a styrofoam box, the straws which included the

sperm-extender mixture were kept 3cm above the liquid nitrogen for 10 minutes. At the end of this period, the straws were immersed in liquid nitrogen at -196°C and kept in a liquid nitrogen tank until further use.

Thawing and Fertilization. The straws were thawed in a water bath at 35°C for 10 seconds. Eggs obtained by abdominal massage from three female fish were pooled. About 200 eggs from this pool were transferred to a sterile petri dish and the sperm sample from the water bath (8 straws) was added and gently mixed for 1 minute. The sperm-egg ratio was determined as $3x10^6$ sperm/egg. Finally, 25 ml of a stock fertilization solution (3g urea, 4g NaCl, 1l distilled water) was added to the sperm-egg mixture and held for 45 minutes. The fertilized eggs in the petri dish were then slowly washed with hatchery water and transferred to hatching trays. The embryos were incubated in hatchery water at 10 ± 1°C and the eyed-embryo rate was determined. Fertilization experiments were carried out using three replicates per group.

Results

The mean values with a 95% Confidence Interval (CI) are presented in all figures and mean ± standard deviation is described. ANOVA was applied to compare spermatozoa motility and motility duration before and after cryopreservation, and Tukey's honest significant difference (HSD) test was used to identify differences among sub-groups.

For all groups, significant decreases in motility, and motility duration of fresh spermatozoa were observed after freeze-thawing. (Figs. 1A,1B). Both motility, and motility duration, of sperm samples was highest before cryopreservation and in the control group. The lowest motility and motility duration was observed in the frozen-thawed sample group treated with the highest concentration of streptomycin. There were significant differences in motility percentages ($p<0.05$) between all sperm samples (Fig. 1A; fig. 1B). There was no significant difference ($p>0.05$) between the other groups treated with streptomycin. The highest fertilization rate was found in the fresh sperm (at the eyed embryo stage) and the control ($p<0.05$). There was no significant difference ($p>0.05$) in the fertilization rate between the streptomycin groups (Fig. 1C).

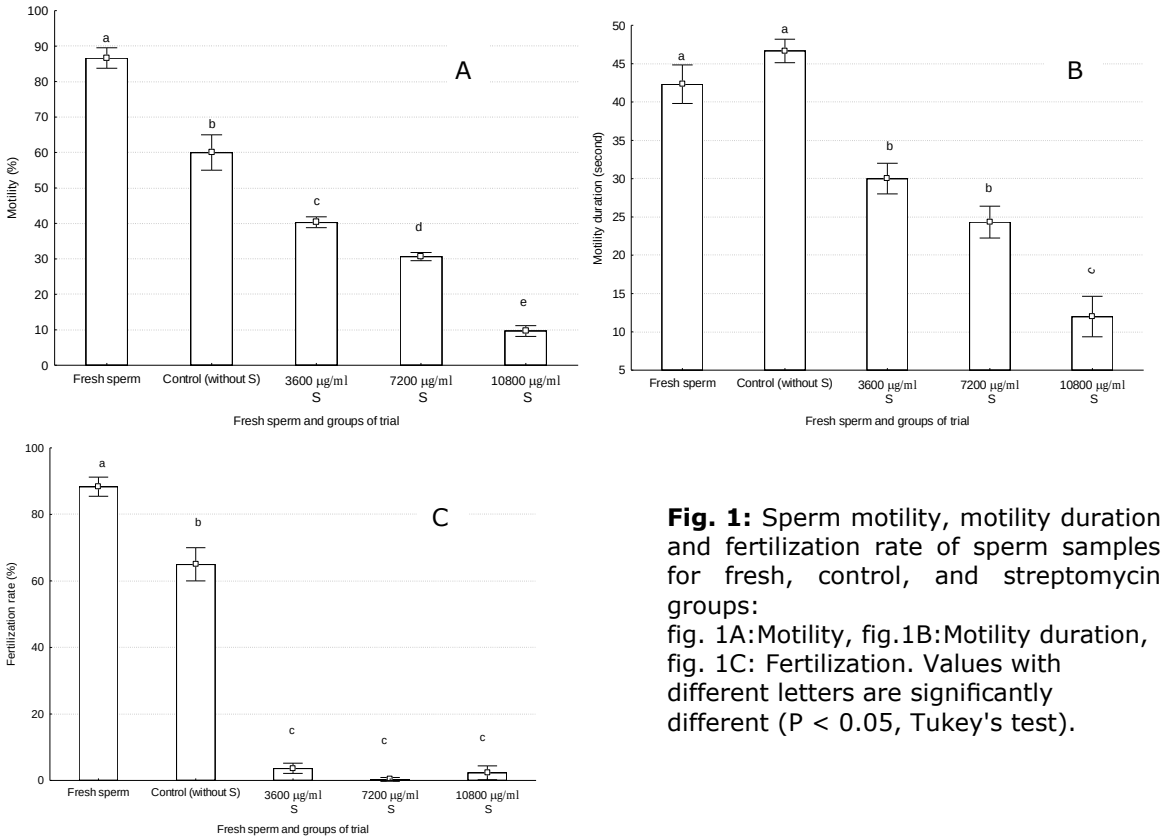

Fig. 1: Sperm motility, motility duration and fertilization rate of sperm samples for fresh, control, and streptomycin groups:
fig. 1A:Motility, fig.1B:Motility duration, fig. 1C: Fertilization. Values with different letters are significantly different ($P < 0.05$, Tukey's test).

Discussion

In fish sperma, antibiotics are used to prevent feces sourced bacterial and fungal contamination which may occur during stripping (Stoss et al., 1978; Stoss and Holtz, 1983). Although antibiotics inhibit bacterial growth, 9000 IU penicillin and 9000µg/ml streptomycin were found to have a deleterious effect on rainbow trout spermatozoa (Stoss et al., 1978). For this reason we used lower concentrations of streptomycin. After the freezing-thawing process we found that the streptomycin reduced the motility of spermatozoa. This result is thought to be due to the change in temperature of crystallization (Salvetti et al., 2006).

Streptomycin and penicillin antibiotics were used in sperm cryopreservation studies of mammals (Chaudhari and Mshelia, 2002; Uysal et al., 2007; Reddy et al., 2010). These studies focused on understanding the effectiveness of diluents rather than researching the effect of antibiotics on the sperm. In a study on Atlantic salmon sperm, 125µg/ml streptomycin, and 125 IU penicillin, was added to the sperm. The spermatozoa maintained motility for 10 days at 0°C under aerobic conditions (Stoss and Refstie, 1983).

The addition of antibiotics to extenders increased the survival rate of sturgeon spermatozoa (Billard et al.,2004). After the addition of 5000 IU penicillin and 5 mg/ml streptomycin to paddle fish sperm kept at 1ºC for 25 days, fertility was 73%.

The use of antibiotics (streptomycin, penicillin) in extenders may cause changes in the amount of ice shaping and on the crystallization temperature of rabbit sperm. This change depends on the extender used (Salvetti et al., 2006). In our study we found that rainbow trout spermatozoa motility decreased after using streptomycin. Further research on the effects of different extender compositions containing streptomycin on spermatozoa motility is needed.

This study showed that the addition of 3600, 7200 and 10800µg/ml streptomycin diminished both motility and motility duration of rainbow trout spermatozoa after freezing-thawing.

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