Effect of Different Extenders and Storage Periods on Motility and Fertilization Rate of Rainbow Trout (Oncorhynchus Mykiss) Semen

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Abstract The effects of different extenders and storage periods on the motility and fertilization rate of rainbow trout (Oncorhynchus mykiss) semen were evaluated after short-term storage. Semen was collected from anesthetized males by the abdominal massage. After determination of main semen characteristics, the pooled ejaculates were diluted with 4 different extenders at a ratio of 1:3, and stored at 4ºC for 72 h. During preservation, spermatozoa motility (%) were determined every 12 h. Fertilization was carried out using the dry fertilization technique. The sperm-egg ratio was approximately 0.5x10^6 sperm/egg for optimum fertilization success. The highest motility (64.4±5.27%) and fertilization rate (94.3 ± 0.58 %) were obtained from semen stored with glucose based extender after 72 h storage. These results indicate that glucose based extender is a better preservative than the other solutions used in the study for the short term preservation of rainbow trout semen.

Keywords Rainbow Trout, Extender, Semen, Short-Term Storage, Fertility

1. Introduction

Preservation of fish sperm for short-term duration is generally useful from the commercial point of view and facilitates various hatchery operations. The short-term storage of sperm at low temperature (4°C) is mostly applied in short-distance transport of gametes collected in different locations, in synchronizing the timing of obtaining good quality of gamete collection from males and females during artificial insemination, in avoiding the aging of sperm, in facilitating hatchery operations, also in experimental programs for genetic studies [1-3].

As seen in previous studies, despite the use of various diluents to preserve fish sperm without reducing fertilizing capacity, results showed that sperm motility, motility duration, viability and fertilizing capacity vary widely. Individual variation, collecting method and storage conditions affect the success in fish sperm dilution [13]. The goal of this study was to identify the effect of cold storage of
sperm from rainbow trout by assessing sperm motility and its insemination ability using different extenders.

2. Materials and Methods

2.1. Broodstock Care and Collection of Sperm and Eggs

The experiment was carried out at the Recep Tayyip Erdoğan University, Iyidere Fisheries Research Centre (IFRC), Rize, Turkey. The broodstock were held in a circular fiberglass tank under a natural illumination and fed with a commercial trout diet at 2% of their body weight per day. The average water temperature was measured as 12.9±0.58 °C (8.0-18.5 °C) during spawning season. A total of 6 mature rainbow trout males (total weight 2536 ±316.3g, total length 56.7 ± 2.82 cm) were randomly selected from broodstock and were used as semen donors in the middle of the spawning season. Fish were fasted for two days prior to collection of semen, anaesthetized in 30 ppm of benzocaine and their abdomens were dried before stripping in order to avoid contamination of semen with urine, mucus and blood cells. The semen was collected into 10 ml graduated glass tubes by gentle abdominal massage. Each male was stripped once only and the total amount of expressible milt was collected individually and the sperm volume was expressed as ml. The semen samples were held on crushed ice (4°C) before analysis, which was undertaken within 4 h of stripping. Eggs were gathered from 5 mature females without anesthesia.

2.2. Evaluation of Sperm Density and Spermatocrit

Sperm density was determined according to the haemacytometric method [14]. Semen was diluted by pipetting 10 μl semen into 990 μl 0.7% NaCl solution. One droplet of diluted semen was placed on a hemocytometer slide (depth 0.1 mm) with a coverslip, the sperm was allowed to settle for 3-5 min, sperm cells were counted using light microscopy (x 40), and spermatooza density was expressed as x 10⁶ cells/ml. Spermatocrit was defined as the ratio of white packed material volume to the total volume of semen multiplied by 100 [15]. Heparinized microhematocrit capillary tubes (75 x 1.1-1.2 mm) were filled with semen and one end was sealed with clay. The capillary tubes were centrifuged at 10,000 rpm for 10 min.

2.3. Evaluation of Sperm Motility, Motility Duration and Ph

The motility of fresh spermatooza from each male was determined immediately after semen was collected. The percent of spermatooza exhibiting rapid, vigorous, forward movement was determined subjectively under a microscope (x 400 magnification) by diluting the semen in activation solution (0.3% NaCl) at a ratio of 1:100 (1 μl sperm to 99 μl activation solution). Motility duration was assessed using a sensitive chronometer (1/100) that was started simultaneously with the addition of activation solution into the samples. Sperm motility observations were done using three replicates per sample. All of the experiments were performed at 17-20°C and all of measurements were carried out by the same investigator under the same conditions for avoiding subjective errors. pH was measured by using indicator papers (Merck 6.4-8).

2.4. Selection of the Sperm Extenders

Four extenders containing calcium chloride (E1), magnesium chloride (E2), glucose (E3) and calcium+magnesium chloride (E4) [16] were selected for evaluation as potential extenders for rainbow trout sperm. The chemical composition of each extender is shown in Table 1. The semen and extenders were maintained in a refrigerator before dilution. For selecting the most suitable extender, the semen was mixed with extender solution at a ratio of 1:3 and placed into 2 ml small plastic vials that were three replicates per treatment. After a rapid shaking, the vials were stored in a refrigerator at 4°C. The samples were analyzed by 12 hours intervals.

2.5. Fertilization

Eggs were pooled from 5 females. Fertilization took place in dry plastic dishes using dry fertilization technique and a portion of ca. 100 eggs (10 g) was placed into each dish. The egg groups were fertilized with cold preserved semen for 72 h storage. Eggs and sperm cells were gently mixed for 10 s. The proportion of sperm-egg was approximately 0.5x10⁶ sperm/egg for optimum fertilization success [17]. After fertilization, 25 ml of 0.3% sodium chloride was added to the sperm-egg mixture as fertilization solution and left for 45 min for swelling of eggs. Subsequently, the fertilized eggs were rinsed with hatchery water (10°C) and placed into vertical incubation trays. Unfertilized and dead eggs were counted and removed continuously. The fertilization rate was determined with the percent of eyed-egg 21 days after insemination.

2.6. Statistical Analysis

All measurements were done in triplicate for each sample and the average of three measurements was used in
subsequent statistical analyses. Data were expressed as means±SD. Motility data were normalized through arcsine transformation. Pearson correlation analysis was used to estimation with spermatologic parameters. Differences between parameters were analyzed by one-way analysis of variance (ANOVA). Significant means were subjected to a multiple comparison test (Tukey HSD) for post-hoc comparisons at a level of $\alpha = 0.05$. All analyses were carried out using SPSS 15.0 for Windows statistical software package.

3. Results

Spermatologic parameters of fresh semen from rainbow trout are shown in Table 2. The volume of semen collected ranged from 10.4 to 21.8 mL. The sperm concentration in the seminal plasma varied between individuals and ranged from $1.6 \times 10^9$ to $15.4 \times 10^9$ sperm cells per milliliter of semen. The semen pH ranged from 7.0 to 7.7, and motility ranged from 80 to 100 per cent.

The effects of extenders and storage periods up to 72 h at 4ºC on motility are shown in Table 3. All semen samples were stored in the refrigerator (4ºC) for 72 h and the motility drastically declined during storage period. The mixing of fresh semen with the glucose based extender solution (E3) significantly ($P < 0.05$) increased the motility of spermatozoa. In contrast, the semen mixed with calcium chloride (E1), magnesium chloride (E2), calcium+magnesium chloride (E4) and the semen stored without the extender (control) had a greatly reduced sperm motility.

The effect of extenders on fertilization rates are shown in Table 4. The differences between the groups were statistically significant ($p < 0.05$). The highest fertilization rate (94.3±0.58%) was obtained with glucose-based extender.

### Table 2. Average spermatologic parameters of rainbow trout semen (n = 6)

| Parameters          | Min   | Max   | Means | SD  |
|---------------------|-------|-------|-------|-----|
| TL                 | 53.0  | 61.0  | 56.7  | 2.82|
| BW                 | 2064  | 2919  | 2536  | 316.3|
| Volume (mL)        | 10.4  | 21.8  | 17.0  | 4.56|
| Spermatocrit (%)   | 25.0  | 37.6  | 33.0  | 4.28|
| pH                 | 7.0   | 7.7   | 7.4   | 0.28|
| Motility (%)       | 80    | 100   | 96.7  | 8.16|
| Duration of motility (s) | 85    | 206   | 121   | 43.9|
| Density (x 10^9)   | 1.6   | 206   | 121   | 43.9|

The effect of extenders on fertilization rates are shown in Table 4. The differences between the groups were statistically significant ($p < 0.05$). The highest fertilization rate (94.3±0.58%) was obtained with glucose-based extender.

### Table 3. Effect of extenders and storage periods on motility rates.

| Storage period (h) | E1    | E2    | E3    | E4    | Control |
|--------------------|-------|-------|-------|-------|---------|
| 12                 | 79.4±6.82a | 82.2±5.65a | 93.3±4.33b | 82.8±5.65b | 87.7±6.18b |
| 24                 | 68.9±7.41a | 74.4±9.17a | 86.1±4.17b | 67.8±4.41a | 82.8±5.65b |
| 36                 | 56.7±7.50a | 59.4±5.27b | 83.3±5.59b | 53.9±4.86b | 77.2±6.18b |
| 48                 | 53.3±4.33a | 55.6±4.64a | 73.3±5.00b | 44.4±5.83a | 56.7±5.59a |
| 60                 | 47.2±9.05a | 48.3±7.07a | 67.8±7.95b | 42.2±3.63a | 48.3±6.12a |
| 72                 | 23.9±4.86c | 22.8±4.41c | 64.4±5.27b | 22.2±3.63c | 26.7±5.61c |

Different superscripts in a row indicate significant differences at $p < 0.05$.

### Table 4. Effect of extenders on fertilization rates of rainbow trout sperm.

| Extenders | Fertilization rates (%) |
|-----------|-------------------------|
| E-1       | 66.0±8.54a               |
| E-2       | 90.3±1.53ab              |
| E-3       | 94.3±0.58b               |
| E-4       | 91.0±2.00ab              |
| Control   | 85.7±2.52ab              |

Different superscripts in a column indicate significant differences at $p < 0.05$. 
4. Discussion

In comparison to other salmonid species (Table 5), the sperm volume found in this study was high compared to *S. trutta fario* [18], *O. mykiss* [19], *S. trutta abanticus* [10], *S. trutta macrostigma* [20], *S. coruhensis* [12] but was similar compared to *O. mykiss* [21]. Sperm density recorded for *O. mykiss* was lower than values observed in *S. trutta abanticus* [10] and *S. coruhensis* [12] but higher than values found in *O. mykiss* [19], *S. trutta caspius* [22], *S. trutta macrostigma* [20]. After freshwater activation, 96.7±8.16% of spermatozoa were motile, higher than in *O. mykiss* [19, 22, 23], and *S. trutta macrostigma* [20].

| Species                  | Volume (ml) | Density (×10⁹/ml) | Motility (%) |
|--------------------------|-------------|-------------------|--------------|
| *S. trutta fario* [18]   | 3.9±1.48    | -                 | -            |
| *O. mykiss* [19]         | 1.22±0.22   | 6.06±0.90         | 73.25±5.15   |
| *O. mykiss* [21]         | 18.17±2.74  | -                 | 72.29±10.79  |
| *O. mykiss* [23]         | -           | -                 | 78.25±3.63   |
| *S. trutta abanticus* [10]| 7.4±0.3     | 17.9±0.4          | -            |
| *S. trutta macrostigma* [20]| 13.93±0.84 | 6.02±0.46         | 80.37±2.36   |
| *S. coruhensis* [12]     | 1.6±0.64    | 13.0±4.93         | -            |
| *S. trutta caspius* [22] | -           | 3.3               | -            |

Spermatozoa that were immotile in the seminal fluid were rapidly activated in contact with fresh water and remained motile for 85-206 s, similar to values reported by Büyükhatipoğlu and Holtz [6], Babiak et al. [24], and Tekin et al. [25], but higher than reported by Bozkurt et al. [21] and Tusset et al. [26] as 78-174 and 22-33 s, respectively. The differences in the properties of sperm may be due to differences in breed, biological characteristics and rearing conditions of brooders, artificial induction of spawning and spawning season.

Short-term storage of sperm using different extenders has been reported in *Oncorhynchus mykiss* [9], *Cyprinus carpio* [27], *Clarias gariepinus* [13], *Salmo trutta abanticus* [10] and *Salmo coruhensis* [12]. In the study, sperm motility was affected during preservation and the proportion of motile cells decreased as the length of storage increased in all groups. The best motility results were obtained with glucose based extender (E3). Similar results for the motility parameters of cold preserved spermatozoa were reported in fish in some experiments [10, 12, 28].

The fertilization results were quite high except for extender E1. Although extenders E2 and E4 were suitable for short-term preservation of rainbow trout sperm, the fertilization rates were all lower than those obtained with extender E3 which gave the best fertilization rates. The reason for the differences among groups in fertilization rate can be explained with decrease in spermatozoa motility. Furthermore, the fertilization can be affected by factors such as dilution ratio of sperm, extender composition, and egg quality [27].

In this investigation, it has been shown that the effect of storage time and extender on motility and fertilization rate was significant (p < 0.05). The fertilization success obtained when extender E3 used as a sperm diluent was significantly better (p < 0.05) than all the other diluents tested. Extender E3 was the most suitable diluent for cold storage of rainbow trout semen at 4°C.

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