



Latin American Journal of Aquatic Research

E-ISSN: 0718-560X

lajar@ucv.cl

Pontificia Universidad Católica de Valparaíso
Chile

Ubilla, Andrea; Valdebenito, Iván

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Latin American Journal of Aquatic Research, vol. 39, núm. 2, julio, 2011, pp. 338-343

Pontificia Universidad Católica de Valparaíso
Valparaiso, Chile

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Research Article

Use of antioxidants on rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792) sperm diluent: effects on motility and fertilizing capability

Andrea Ubilla¹ & Iván Valdebenito¹

¹Universidad Católica de Temuco, Facultad de Recursos Naturales
Escuela de Acuicultura. P.O. Box 15-D, Temuco, Chile

ABSTRACT. The present investigation determined how different antioxidants incorporated into the sperm diluent for cold storage of semen affected sperm motility and spermatozoan fertility capabilities of the rainbow trout. For the evaluations, fresh semen (C) and semen that had been stored without diluents (T1) were used as control groups. The diluents were prepared using a base of UCT diluents (T2), adding grape polyphenol (0.1 g 100 mL⁻¹) (T3), trolox C (0.1 g 100 mL⁻¹) (T4), polyphenol (0.1 g 100 mL⁻¹) plus trolox (0.1 g 100 mL⁻¹) (T5), and vitamin C (0.018 g 100 mL⁻¹) (T6). The incorporation of antioxidants into sperm diluents prolongs motility and fertility of rainbow trout semen. The results show that by day two, all of the treatments showed level 5 sperm motility. After seven days of storage, only T3 and T6 dropped to level 4 sperm motility. The duration of flagellate activity on this day was maximal for T3 with 36.87 ± 0.51 s and minimal for T6 with 29.78 ± 0.52 s. On day seven, fertility was maintained with no statistically significant differences between the control and T2 ($92.80 \pm 0.62\%$), T3 ($83.66 \pm 2.52\%$), T4 ($90.46 \pm 1.60\%$), T5 ($83.57 \pm 2.75\%$), and T6 ($83.57 \pm 2.30\%$). By days 10 and 17 of storage, the fertility of T1 was zero and that of T2 was significantly lower than the control group. On day 17, the highest percentage of fertilization was $97.38 \pm 1.85\%$ for T5 and the lowest value was $64.69 \pm 3.76\%$ for T2. The results allow concluding that the sperm viability of semen stored with different antioxidants is significantly prolonged.

Keywords: *Oncorhynchus mykiss*, polyphenol, trolox C, vitamin C, fertilizing capability, sperm motility.

Uso de antioxidantes en el diluyente espermático para trucha arcoiris *Oncorhynchus mykiss* (Walbaum, 1792): efecto en la motilidad y capacidad fecundante

RESUMEN. En la presente investigación se determinó el efecto en la motilidad espermática y la fertilidad del espermatozoide de trucha arcoiris, de diferentes antioxidantes incorporados en el diluyente espermático para el almacenamiento en frío de semen. Para las evaluaciones se utilizó como control semen fresco (C) y semen almacenado sin diluir (T1), los diluyentes fueron preparados utilizando como base el diluyente UCT (T2) al que se le incorporó polifenol de uva (0,1 g 100 mL⁻¹) (T3), trolox C (0,1 g 100 mL⁻¹) (T4), polifenol más trolox (0,1 g 100 mL⁻¹ + 0,1 g 100 mL⁻¹), respectivamente (T5) y vitamina C (0,018 g 100 mL⁻¹) (T6). La incorporación de antioxidantes en los diluyentes espermáticos, prolongan la motilidad y fertilidad del semen de trucha arcoiris. Los resultados muestran que al día dos todos los tratamientos presentaban un nivel 5 de motilidad. Después de 7 días de almacenamiento, sólo T3 y T6 bajaron a un nivel 4 de motilidad. La duración de la actividad flagelar en este día fue máxima en T3 con $36,87 \pm 0,51$ s y mínima en T6 con $29,78 \pm 0,52$ s. Al día 7 la fertilidad se mantuvo sin diferencias estadísticamente significativas con el control en T2 ($92,80 \pm 0,62\%$), T3 ($83,66 \pm 2,52\%$), T4 ($90,46 \pm 1,60\%$), T5 ($83,57 \pm 2,75\%$) y T6 ($83,57 \pm 2,30\%$). Los días 10 y 17 de almacenamiento, la fertilidad de T1 fue cero y la de T2 fue significativamente menor al control. Al día 17 el mayor porcentaje de fecundación fue de $97,38 \pm 1,85\%$ para T5 y el menor valor fue de $64,69 \pm 3,76\%$ para T2. Los resultados permiten concluir que el semen almacenado con distintos antioxidantes prolonga significativamente la viabilidad espermática.

Palabras clave: *Oncorhynchus mykiss*, polifenol, trolox C, vitamina C, capacidad fecundante, motilidad espermática.

INTRODUCTION

Sperm extender diluents improve motility and fertilization percentages of cold stored semen (Stoss, 1983; McNiven *et al.*, 1993; Billard, 1990; Sánchez & Rubilar, 2001; Valdebenito *et al.*, 2009). These solutions prevent the oxidization of the plasmatic and mitochondrial membrane, delivering energetic substrates and allowing the maintenance of spermatozoa at stable pH and osmolarity conditions of the extracellular mediums (Morisawa & Susuki, 1980; Morisawa *et al.*, 1983; Batellier *et al.*, 2001). Gametes are aerobic cells where oxygen plays an essential role for their performance. Oxygen free radicals are continuously generated in the different metabolic pathways, and are capable of interacting with different biomolecules, causing cell damage. Oxidative stress is a consequence of the unbalance produced between the production of free radicals and the anti oxidizing capabilities of an organism, where this can become very harmful if there is a high production of oxygen reactive species (ORS) (Saleh *et al.*, 2002; Membrillo *et al.*, 2003).

In mammals, sperm motility can become seriously affected by ORS's. At a molecular level they can obstruct the phosphorylation of proteins, preventing sperm movement (De Lamirande *et al.*, 1998). Reactive species also exist, that are produced naturally by the cell, these are in low concentrations intervening in processes like capacitation, acrosomic reaction and the union of the spermatozoa to the pellucid zone (Sharman & Agarwal, 1996; De Lamirande *et al.*, 1997, 1998; Aitken *et al.*, 2004; Allamaneni *et al.*, 2004).

Antioxidants are molecules that prevent the uncontrolled production of free radical or inhibit their reactions with biological structures (Halliwell & Chirico, 1993). In seminal plasma and in spermatozoa of teleostei fish there are different types of antioxidants (Liu *et al.*, 1995; Ciereszko *et al.*, 2000; Lahnsteiner *et al.*, 2010a), which are important for the *in vivo* maintenance of sperm viability, and that could have relevance for the practice of supplementation of the semen storage and cryopreservation mediums to improve the quality of the spermatozoa. Ascorbic acid (Ciereszko & Dabrowski, 1995; Metwally & Fouad, 2009) and Uric acid (Ciereszko *et al.*, 1999) are considered important antioxidants in teleostei semen. In rainbow trout and carp semen, methionine sulfoxide reductase and methionine can have antioxidant functions (Lahnsteiner, 2009).

Experimentally, to reduce the levels of ORS in mammals, ascorbic acid and/or catalase have been added to sperm extender diluents, where satisfactory

results have been obtained (Ball *et al.*, 2001a), since the enzymes present in seminal plasma are not always efficient enough to reduce the effects of ORS's (Lamirande *et al.*, 1997; Pecker *et al.*, 1997).

The objective of this present investigation was to evaluate the effects of the incorporation of three antioxidants applied to semen of rainbow trout (*Oncorhynchus mykiss*) during cold storage for 17 days.

MATERIALS AND METHODS

Gamete recollection and semen storage

To simulate the conditions of the fish farms, a "pool" of semen was used (1 mL per male and six ml total) with maximum motility was obtained from six adult male rainbow trouts (*Oncorhynchus mykiss*) that were at their second spawning from the Quetro S.A. fish farm (33°23'32''S, 71°40'47''W) in the south of Chile. The semen samples were obtained by abdominal massage, avoiding contamination with feces and urine. An initial motility evaluation was carried out at the same center, according to the Sánchez-Rodríguez & Billard (1977) scale, that considers zero as the minimum and five as the maximum score. The selected samples (all with level 5) were taken to the Reproduction Laboratory of the Universidad Católica de Temuco (UCT) in sterilized containers with oxygen, at 4°C and in the absence of light.

Sperm density

Sperm density of the semen (N° of spermatozoa/mL), was determined in four aliquots, by a counting Neubauer chamber using the methodology described by Oppenheim (1973) for blood cells.

Sperm count

Sperm count was determined in five samples. Semen was put into five microhematocrit. Then, these were centrifuged at 10.000 rpm for a 10 min period. Later, by means of a table, the percentage of spermatozoa was determined from the semen samples.

Sperm diluents

To evaluate the effectiveness of the antioxidants polyphenol, trolox C and vitamin C, one part of the pool semen was diluted into two parts of diluent (1:2) according to the following treatment group: fresh semen control (C) of different male; undiluted stored semen (T1); semen diluted with UCT medium (UCT® with KCl to inhibit motility) (T2); T2 + polyphenol (0.1 g 100 mL⁻¹) (T3); T2 + trolox C (0.1 g 100 mL⁻¹)

(T4); T2 + polyphenol ($0.1 \text{ g } 100 \text{ mL}^{-1}$) + trolox C ($0.1 \text{ g } 100 \text{ mL}^{-1}$) T5; T2 + vitamin C ($0.018 \text{ g } 100 \text{ mL}^{-1}$) (T6). Osmolarity was determined same day using a Micro-Sample Osmometer model Fiske® 210 and the pH values were measured with a digital pHmeter model pH 21 and the value that were obtained are indicated in Table 1.

Evaluation of the level and duration of sperm motility of rainbow trout semen with different sperm diluents

Once at the reproduction laboratory, the semen samples were diluted at a 1:2 ratio with the applied diluents and maintained at 4°C in an incubation chamber, with permanent agitation and in the absence of light.

For sperm activation, an activating solution with $280 \text{ mOsmol kg}^{-1}$ osmolarity and 9.0 pH level, prepared with $\text{NaCl } 9\%$ was used. The evaluations were carried out in the reproduction laboratory of the UCT at room temperatures (10°C), where flagellate activity of the spermatozoa was observed through a Nikon Eclipse E400 microscope; at $10\times$ to determine the level of motility, and at $40\times$ to determine the duration (in seconds) of this flagellate activity, considering the moment the spermatozoa initiate linear movement and ending when they begin local rotation movements. The evaluation was carried out in 20 aliquots for each treatment. These evaluations were carried out on days 2, 4, 7, 9, 11, 14 and 17 of storage.

Fertilizing capabilities

The evaluation of the fertilizing capability was done after 7, 10 and 17 days of storage, on a “pool” of recently extracted fish eggs, from six females during their second spawning, raised at the same fish farm. The eggs were deposited into plastic containers (five replicas per treatment) with approximately 100 eggs each. Coelomic fluid was extracted and fertilization was carried out by adding $10 \mu\text{L}$ of semen from the control (C) and T1 group. For diluted semen of group T2, T3, T4, T5 and T6, $20 \mu\text{L}$ were used for each replica.

Five minutes after fertilization, river water was added to the gametes and semen residues were cleaned. Then, hydration of the eggs was carried out for 30 min followed by the introduction of the eggs into enumerated micro-incubators and into containers with an open flow system at 9°C , according to the fish farm protocols.

Determination of fertilizing percentages

After 14 days of incubation, the percentage of fertilization was determined using acetic acid 10% , by depositing 30 random fish eggs and considering those that have observed neural tubes as fertilized eggs.

Statistical analysis

To establish the level of motility of the semen, the mode of each treatment was used. For the rest of the parameters, the average values and standard deviations were used. Using the statistical software GaphPad Prism 5, an ANOVA and Kruskal-Wallis tests were applied to identify the existence of statistically significant differences between the group ($P \leq 0.05$).

RESULTS

Sperm motility and sperm count

The sperm density obtained from the “pool” of semen was of $16 \pm 2,3 \times 10^9$ spermatozoa/mL and the average percentage of the sperm count obtained during the experiment was of $33,2 \pm 1,1\%$. The mode that was registered for motility on day 2 in all of the treatment group was level 5. On day 7; T3 and T6 had lowered to level 4. By day 9; T1 and T2 had level 0 and on day 17; T3, T4, T5 and T6 maintained level 3 (Fig. 1).

The duration of motility observed on day 2 (Fig. 2) was of $37.07 \pm 0.24 \text{ s}$, for T4 and the minimum value of $34.04 \pm 0.35 \text{ s}$ for T5. On day 17 T1 and T2 did not present flagellate activity, the highest value was of $7.08 \pm 1.02 \text{ s}$ for T5 and the lowest was of $2.34 \pm 0.48 \text{ s}$ for T3. The differences that were observed between both treatment group were statistically significant.

Fertilizing capabilities

The sperm density that was used for fertilization was of $1.600.000$ spermatozoa/oocyte in all of the treatment group. The average fertilization percentages on day 7 were $83.15 \pm 6.10\%$ for C and $66.42 \pm 4.06\%$ for T1 (Fig. 3). Treatments T2, T3, T4, T5 and T6 did not present statistically significant differences. On day 10, T1 presented 0% of fertilization and T2 presented $68.02 \pm 1.40\%$. T3, T4, T5 and T6 did not present statistically significant differences. By day 17, T1 did not present fertilization, and T2 presented $64.69 \pm 3.76\%$. T3, T4, T5 and T6 did not present statistically significant differences in their fertilizing capability (Fig 3).

DISCUSION

The results of the present investigation show that the use of vitamin C, trolox and polyphenol in sperm diluents for rainbow trout, preserves sperm motility, improving significantly the fertilizing capability of semen and increasing spermatozoa storage times.

Table 1. Osmolarity and pH values for the treatments used in the storage of rainbow trout semen.**Tabla 1.** Valores de osmolaridad y pH para los tratamientos utilizados en el almacenamiento de semen de trucha arcoiris.

Treatments	Information	Osmolarity mOsmol kg ⁻¹	pH
C	Fresh semen	300	8.0
T1	Undiluted stored semen	300	8.0
T2	Semen diluted with UCT medium	288	8.0
T3	T2 + polyphenol	280	7.5
T4	T2 + trolox	290	7.8
T5	T2 + polyphenol + trolox	288	7.7
T6	T2 + vitamin C	260	7.5

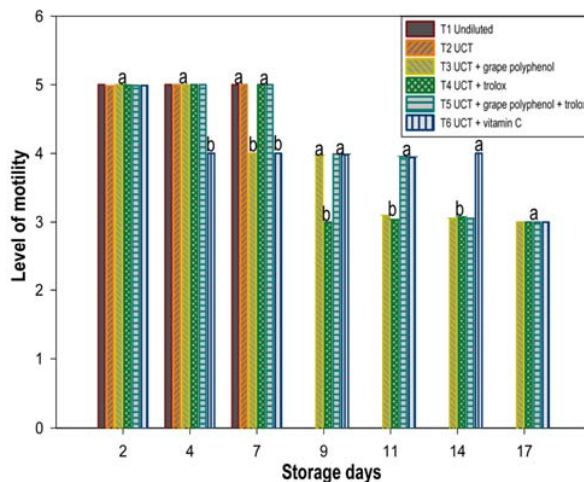
**Figure 1.** Level of motility (Mode) in rainbow trout spermatozoa, submitted to different antioxidant treatments. Different letters seen a same day indicate that statistically significant differences exist ($n = 20$).

Figura 1. Nivel de motilidad (Moda) en espermatozoides de trucha arcoiris sometidos a diferentes tratamientos con antioxidantes. Letras diferentes en un mismo día indica diferencias estadísticamente significativas ($n = 20$).

Sperm motility in all the antioxidant treatment group is considerably improved in T5, which presents the highest value of 7.08 ± 1.02 s at day 17, this can be explained since the combined use of polyphenol and trolox can increase their actions as antioxidants (Ball *et al.*, 2001b). Polyphenols on their part, act mainly by chelating metals and capturing *in vitro* the ORS's and nitrogen reactive species (NRS) in a more efficient manner than vitamin C (Larkins, 1999). The same occurs with the separate use of trolox and vitamin C, this last element is the main antioxidant found in plasma and in the cells, that by donating electrons to the tocopherol radical of the oxidized vitamin E, it recycles the antioxidant function of α -tocopherol,

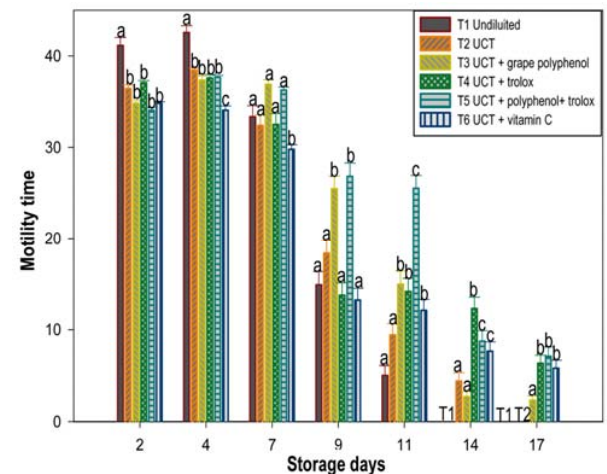
**Figure 2.** Motility (s) of rainbow trout spermatozoa submitted to different antioxidant treatments. The values are given as averages \pm SD ($n = 20$). Different letters indicate that statistically significant differences exist for a same day ($P < 0.05$).

Figura 2. Motilidad (s) en espermatozoides de trucha arcoiris sometidos a diferentes tratamientos con antioxidantes. Los valores se entregan en promedio \pm SD ($n = 20$). Letras diferentes indica que existen diferencias estadísticamente significativas para un mismo día ($P < 0,05$).

helping to protect the lipid membrane from peroxidation (May, 1999). α -tocopherol can act as an antioxidant or pro-oxidant, since it inhibits or facilitates lipid peroxidation of the low density lipoproteins. The pro-oxidizing activity of α -tocopherol is prevented by ascorbate, for which vitamin E can only be effective in combinations with vitamin C (Carr *et al.*, 2000). The combination of vitamin E, a lipophilic antioxidant, with vitamin C, a hydrophilic antioxidant and/or selenium, desintoxicate the lipids from the peroxides (Schwenke & Behr, 1998).

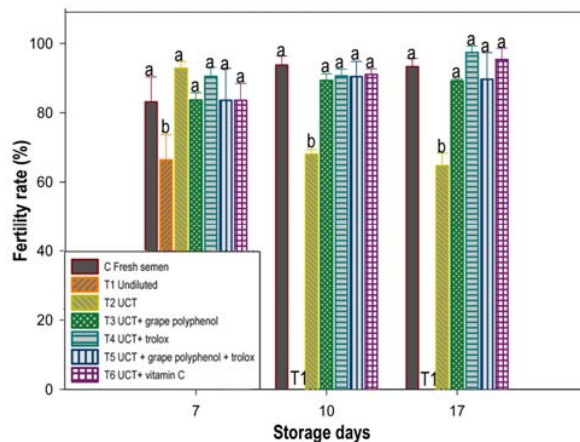


Figure 3. Fertilizing capabilities of rainbow trout spermatozoa submitted to different antioxidant treatments. The values are given as averages \pm SD ($n = 15$). Different letters indicate that statistically significant differences exist for a same day ($P < 0.05$).

Figura 3. Capacidad fecundante en espermatozoides de trucha arcoiris sometidos a diferentes tratamientos con antioxidantes. Los valores se entregan en promedio \pm SD ($n = 15$). Letras diferentes indica que existen diferencias estadísticamente significativas en un mismo día ($P < 0,05$).

Lahnsteiner *et al.* (1997) studied aging in rainbow trout *Oncorhynchus mykiss* semen, during cold storage for 2 h, and he found a decrease in fertilizing capabilities, motility and metabolism of the spermatozoa, regarding recently extracted semen. This decrease was mainly due to the energetic increase that the spermatozoa requires during fertilization and to an increase of polyunsaturated fatty acids, where they become vulnerable to the attack of free radicals.

In the present investigation, variations in motility times were present in the first two days of storage of semen with antioxidants, regarding undiluted semen. On the other hand, this is not reflected for the fertilizing capabilities of the treatment group with antioxidants (T3, T4, T5 and T6) by day 17, which registered high fertilization percentages without significant differences among them. Hoysak & Liley (2000) report that these variations in flagellate activity do not affect fertility, since in sockeye salmon, it only takes 5-10 s for the spermatozoa to have contact with the oocyte to fertilize it.

Lahnsteiner *et al.*, (2010a) and Lahnsteiner *et al.*, (2010b) incorporated different antioxidants for defense from the ORS's into a sperm diluent, finding positive effects on sperm viability (sperm motility, membrane integrity and lipid peroxidation) in stored

semen of different teleostei fish for 72 to 120 h with uric acid and 72 h with methionine.

There is limited information on antioxidant systems in teleostei fish spermatozoa to be able to define which antioxidant is the most adequate one for semen storage. It is because of this, that in this work, the effect of adding antioxidants that are soluble in water and lipids, into the sperm diluents is evaluated to maintain the integrity of the spermatozoa, protecting the cells and reducing the risk of lipid peroxidation which would affect sperm motility (Ciereszco & Dabrowski, 1995).

Due to its positive effects on motility parameters, fertilizing capabilities, stability and low price, the use of polyphenol, trolox and vitamin C can be a useful complement for the storage of salmonid spermatozoa.

ACKNOWLEDGEMENTS

Financed by the FONDEF D06I1020 Project and collaboration of the Quetro S.A. and Dropco S.A. companies.

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