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CRYOPRESERVATION OF RAINBOW TROUT SEMEN: DILUENT, STRAW AND THE VAPOR COLUMN *

[Criopreservação do sêmen de truta arco-íris: diluente, palheta e a coluna de vapor]

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ABSTRACT

The present paper concerns to the development of a simple semen freezing technique to be used in commercial systems. Fresh milt was obtained from two years-old adult males of rainbow trout. The samples were previously cooled (4ºC), then diluted, packaged in straws, and frozen in liquid nitrogen vapor. The freezing system was composed by a 33 L Styrofoam™ box with a graduated metal web; straws of 0.5 mL and 4 mL were tested and two types of extenders were used: V2e with 10% DMSO and modified Cortland solution with 24% DMSO. Two experiments were performed: the first one evaluated the effect of the extenders and straw type on the fertility of the frozen/thawed semen, and the second one verified the effect of the vapor column and straw position on the viability of the frozen/thawed semen, using 0.5 mL-straws and V2e extender. The sperm viability was tested by checking eyed eggs production. In the first experiment, there were no statistical differences (P>0.05) between the two tested straws and the modified Cortland solution. However, the V2e solution treatment showed a significantly higher fertilization rate when associated to 0.5 mL straw. In the second experiment, a remarkable statistical interaction (P>0.05) was observed between the straw cargo and vapor column (freezing point) effects on the percentage of eyed eggs.

Key words*:* fish; spermatozoa; semen; cryopreservation; rainbow trout; *Oncorhynchus mykiss*

RESUMO

A presente investigação visou ao desenvolvimento de uma técnica de congelação de sêmen, simples para ser aplicada em sistemas de produção. O sêmen fresco foi obtido de machos adultos de truta arco-íris com dois anos de idade. As amostras foram previamente resfriadas (4ºC), diluídas, envasadas em palhetas e congeladas em vapor de nitrogênio líquido. O sistema de congelação foi composto de uma caixa de isopor de 33 L e uma estante de metal graduada, utilizando-se palhetas de 0,5 e 4,0 mL e dois tipos de diluentes: V2e com 10% de DMSO e solução modificada de Cortland com 24% de DMSO. Dois experimentos foram realizados: o 1º verificou o efeito dos diluentes e dos tipos de palhetas em relação à fertilidade do sêmen descongelado, o 2º estudou o efeito da coluna de vapor e da carga de palhetas, usando palhetas de 0,5 mL e o diluente V2e, na efetividade do sêmen descongelado. A viabilidade espermática foi testada por sua capacidade de produzir ovos-olhados. No primeiro experimento não houve diferença estatística (P>0,05) entre as duas palhetas testadas em relação à solução modificada de Cortland. Entretanto, o tratamento com a solução V2e demonstrou aumento significativo das taxas de fertilização, quando associado a palheta de 0,5 mL. No segundo experimento ocorreu interação estatística significativa (P>0,05) entre os efeitos da carga de palhetas e da coluna de vapor (ponto de congelação) em relação à porcentagem de ovos-olhados.

Palavras-chave: peixe; espermatozóide; sêmen; criopreservação; truta arco-íris; *Oncorhynchus mykiss*

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Introduction

The cryopreservation of fish semen is an effective way to improve artificial reproduction because it facilitates genetic manipulation and the selection of the broodstock, reducing the amount of male breeders stocks since masculine gametes are available at any time. Furthermore, the cryopreservation allows the establishment of gene banks, useful in hybridization programs and genetic conservation of extinction endangered species.

The best results with semen cryopreservation in salmonids have been obtained at laboratory conditions, employing few semen or ova (JAMIENSON, 1991) and high cost equipment. However, only a few works have reported cryopreservation techniques applied to field conditions where a great volume of semen must be handled (CLOUD; MILLER; LEVANDUSKI, 1990; STEINBERG *et al*., 1995; WEISMANN; LAHNSTEINER; PATZNER, 1995). Moreover, some authors have argued about the lack of consistency in the current methods for short-term preservation and cryopreservation of rainbow trout semen (CONGET *et al*., 1996).

The use of straws for high semen volume should be desirable, but it requires longer diameter straws, that, in turn, increase the freezing time. Nowadays, the most used straws are made of polyethylene, with capacity ranging from 0.25 mL to 0.5 mL (micro and medium straws, respectively). WHELEER and THORGAARD (1991) analyzed a 4.5 mL straw using a simple extender (5.4% glucose, 10% yolk fresh hen's egg, and 9% DMSO) and suggested that it could constitute a routine method for cryopreservation.

CAROSFELD *et al*. (1990) and FOGLI da SILVEIRA *et al*. (1994) cryopreserved semen of *Piaractus mesopotamicus* and *Oncorhynchus mykiss*, respectively, and found an interactive effect between the extender composition and the type of straw on the success of cryopreservation. WEISMANN; LAHNSTEINER; PATZNER (1995) proposed that, in open systems, a 0.5 cm variation of the vapor column (distance between the liquid nitrogen and the straws) significantly decreases the fertility of the thawed semen, and this fact must be considered for routine cryopreservation methods. Moreover, LAHNSTEINER; WEISMANN; PATZNER (1997) showed that the optimal height of the vapor column is a species-dependent trait.

The first objective of the present study was to investigate the effect of the straw volume and the type of extender in the success of rainbow trout semen cryopreservation. Two extenders were used: V2e and Modified Cortland solution, which have been frequently employed in tropical fishes and salmonids at South America. In a second experiment, the interaction effects between straw cargo and height of the nitrogen vapor column were evaluated, as both seem to affect the freezing time and the final quality of spermatozoa.

Material and Methods

Collection of semen and eggs

Pools of semen and eggs of three rainbow trout individuals, *Oncorhynchus mykiss* (2 years-old), belonging to the stock from the Instituto de Pesca, Campos de Jordão - SP, were used per experiment. The oocytes and semen were collected by abdominal pressure. In order to obtain sperm free of urine or feces contamination, the first portion of the released semen was discarded. The spermatozoa motility was around 80% according to SALISBURY and VANDEMARK'S (1964) scale.

Experiment 1

Two extenders, the V2e and a modified-Cortland solution, in two types of straws (0.5 mL and 4.0 mL) (I.M.V., L´Aigle, French) were tested. In each of the four groups, the diluted semen was frozen in 10 straws with 5 cm of vapor column (FOGLI da SILVEIRA *et al.,* 1994). After 48 h, the material was thawed and used for fresh-oocytes fertilization. Two 0.5 mL straws and one 4.0 mL straw were used in order to fertilize 150 and 350 fresh-oocytes, respectively. The fertilization rate was calculated by the percentage of eyed embryos in relation to the total number of eggs.

Experiment 2

The effects of the straw cargo (number of straws: 10 and 15) and the vapor column (3.5 cm, 5.0 cm,

and 6.5 cm) on the quality of cryopreserved semen were tested. These observations were made with 0.5 mL straws and V2e solution, both yielding the best results according to the first study. Two straws were used aiming to fertilize 150 fresh-oocytes in the fertilization test.

Semen cryopreservation and thawing

An open cryopreservation system consisting of a 33 L Styrofoamä box (33.9 cm height x 26 cm width x 41 cm length), with 8.73 L of liquid nitrogen (5 cm height) was used. The extenders and the fresh semen were previously maintained at 4ºC and then diluted (3:1), packaged in the straws, frozen in liquid nitrogen vapor (up to -80ºC), subsequently immersed in liquid nitrogen, and stored at -196ºC. During straw manipulation for freezing, the evaporation of liquid nitrogen required some adjustments in the vapor column.

The thawing process was accomplished by immersing the straws into warm water (70º to 80ºC) for 3 to 5 s (0.5 mL straw), as described by FOGLI da SILVEIRA *et al.* (1994), or for 15 s (4 mL straw).

Extenders

The extenders solutions were prepared as follows:

- V2e Solution (STEIN and BAYRLE, 1978): NaCl = 0.75 g; $NaHCO_3$ = 0.20 g; KCl = 0.038 g; glucose = 0.10 g; distilled water = 100 mL; yolk fresh hen's egg = 20 mL (313 mOsm; pH = 8,0) and 10 mL of dimethyl sulfoxide (DMSO).

- Modified Cortland Solution (CAROSFELD *et al.*, 1990): NaCl = 0.725 g; $CaCl_2.2H_2O$ = 0.023 g; KCl = 0.038 g; NaH^2PO^4 = 0.041 g; $Mg(SO_4).7H_2O$ = 0.023 g; $NaHCO_3$ = 0.10 g; glucose = 0.10 g. distilled water = 100 mL (126 mOsm; pH = 8,3) and 24 mL of DMSO.

Statistical analyses

The mean values showed normal distribution and were compared in a two-way ANOVA (2 x 2 or 2 x 3), followed by LSD post-comparison tests in SAS (α=0.05). All experiments were performed with three replicates.

Results and Discussion

Analyses about the effects of the straw and extenders on the cryopreservation of rainbow trout semen showed statistical interaction (ANOVA: F = 5.61; p = 0.045) and these data are expressed at figure 1. Accordingly, the two types of straws tested with the modified Cortland solution didn't exhibit significant differences (P>0.05). However, significantly higher rates of fertilization were shown when V2e solution was used coupled to 0.5 mL straws instead of 4 mL straws. The increase of fertilization rates detected using a 0.5 mL straw (V2e extender) may be related to the residual energy of the semen at this condition, taking into account that, close to the freezing point, the semen mass releases a high amount of energy, increasing the semen temperature (RICHARDSON *et al*., 1999). Under this condition, a peak of temperature could damage the spermatozoa by forming ice crystals. In this case, the use of middle straws (0.5 mL) with the V2e extender could provide a better freezing condition for gametes, resulting in higher fertilization rates. Why such effect occurred, however, is a question to be solved in future investigations.

In the second experiment, a high statistical interaction was verified between the effects of both straw cargo and vapor column (freezing temperature) on the preservation of spermatozoa characteristics (Figure 2) (ANOVA: F = 7.45; p = 0.008). Although some differences have been detected between the compared conditions, it was consistently demonstrated that the use of 6.5 cm vapor column with 10 straws resulted in the highest percentage of eyed eggs (LSD *post hoc* test). Such effect should determine the optimal freezing conditions for the gametes, as proposed by WEISMANN; LAHNSTEINER; PATZNER (1995). The use of a higher number of straws (15) led to a relative survival of eyed eggs, from 10 to 20% , whereas a lower number of straws (10) in a 6.5 cm vapor column showed higher survival rates (about 35%). Despite this difference in survival rate (about 15%), the use of a higher number of straws in each freezing

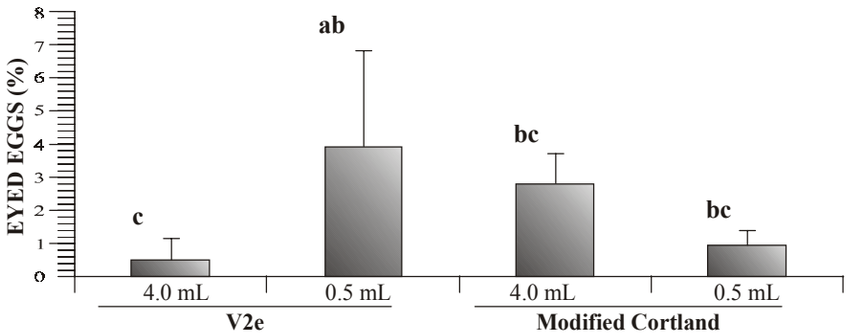

Figure 1. Effects of straw (0.5 mL; 4.0 mL) and extenders (V2e solution and modified Cortland) solution on fertilization of rainbow trout eggs. Mean values (+ standard deviation) were obtained from four (V2e) and two (Cortland) replicates. Mean values sharing, at least, a same letter are not statistically different from each other (ANOVA: F = 5.61, p = 0.045).

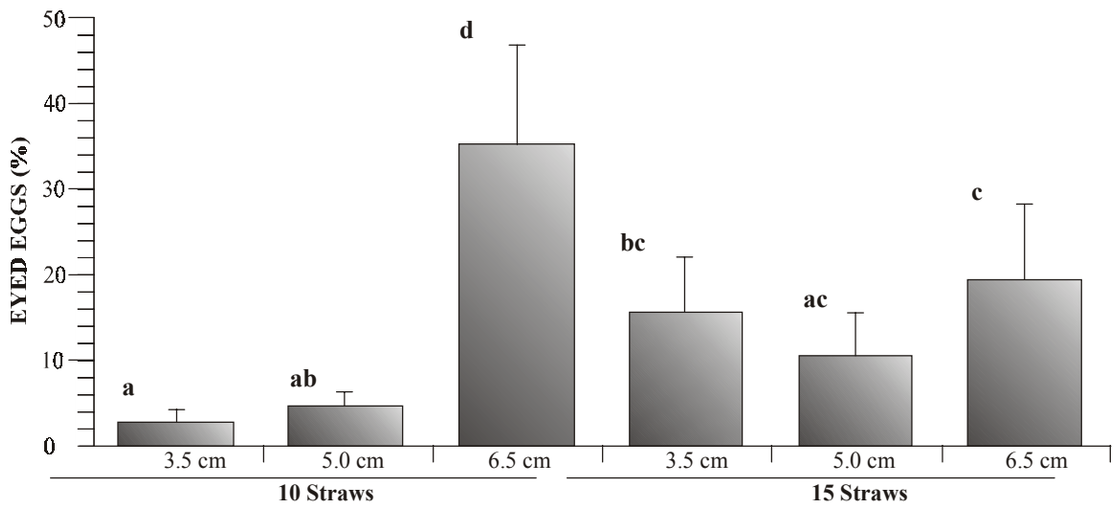

Figure 2. Effects of variable straw cargo (10 and 15 straws) and vapor column (3.5, 5.0, and 6.5 cm) on fertilization in rainbow trout. Mean values (+ standard deviation) were obtained from three replicates. Mean values sharing, at least, a same letter are not statistically different from each other (ANOVA: F = 7.45; p = 0.008).

session saves time, and thus the semen can be frozen within a short period of time. Actually, the storage time prior freezing is an important issue, affecting the post-thawing fertility capacity (HOLTZ, 1993 and WEISMANN; LAHNSTEINER; PATZNER, 1995). Furthermore, the method involving 15 straws also allows a higher range for the vapor column, which is interesting for practical procedures. As previously stated by WEISMANN; LAHNSTEINER; PATZNER, (1995) and LAHNSTEINER; WEISMANN; PATZNER (1997), the present results provide some new interpretation, as, for instance: if the number of straws is increased, the vapor column must be adjusted (decreased) to reach a same freezing rate.

The cryopreservation system used in the present study can be easily handled, and it is highly cost-efficient. A fast and clear determination of the nitrogen level in the tank while handling straws is an issue yet to be accomplished. This process, which takes no more than 1 min, can condense the air moisture into water, disturbing the visualization of nitrogen level.

This study shows that some aspects of the technique for rainbow trout semen cryopreservation, such as the extender type, straw size, and vapor column, might improve the success of fertilization. Thus, the number of straws can be determined by the height of the vapor column.

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