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Potassium ions in extender differentially influence the post-thaw sperm motility of salmonid fish

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ABSTRACT

Potassium ions are known to have an inhibitory effect on the sperm motility of salmonids. For this reason, the addition of K^+ to the extender is frequently applied. However, the effect of the addition of K^+ to the extender has not yet been tested. The aim of this study was to test the influence of potassium ion supplementation of the extender on the sperm motility parameters from five *Salmonidae* species (rainbow trout (*Oncorhynchus mykiss*), sex-reversed female rainbow trout, whitefish (*Coregonus lavaretus*), brown trout (*Salmo trutta*) and brook trout (*Salvelinus fontinalis*)). Semen samples were diluted in extender containing 0.18 M glucose in 9% methanol (GM) supplemented with 0, 20 or 40 mM potassium chloride. After thawing sperm were stored for 30, 60, 120 and 240 min at 4 °C. Our results demonstrated that the presence of potassium ions in the extender had a negative effect on percentage of motile sperm in four of the salmonid species. In contrast, potassium ions appeared to have a positive effect on percentage of post-thaw motile sperm in whitefish semen. However, this effect could be mimicked by changing the osmolality of the extender (which was achieved by increasing the glucose concentration to 0.22 M). The addition of potassium ions turned out to have no positive effect on post-thaw storage time. Our results suggest that osmolality, rather than potassium ions, seems to be essential for cryopreservation success of salmonids sperm. Further studies should focus on the effects of small changes in osmolality on the post-thaw quality of semen.

1. Introduction

Cryopreservation of fish sperm is of interest not only for fish breeding but also for the conservation of genetic resources [8]. Cryopreserved sperm allow for the availability of gametes throughout the year [28], facilitating the ease of transportation of gametes [26] as well as supporting artificial fertilisation when distant periods of maturation between sexes of a same species is often observed. The use of cryopreserved sperm can also reduce animal breeding costs, as the number of individuals in a fish farm can be reduced. Many studies have investigated cryopreservation of sperm from *Salmonidae*; however, different freezing protocols have been used with varying results. For this reason, there is a need for standardisation of the methodological parameters routinely used for cryopreservation of sperm from these fish.

Potassium ions (K^+) are known to have an inhibitory effect on the sperm motility of salmonids. The K^+ concentration that effectively inhibits sperm activation in salmonids has been reported to range between 0.1 mM and 2 mM [13,3]. Therefore, a high potassium concentration is a major inhibitor of sperm motility in salmonids, but the extent of inhibition varies in relation to the time of reproductive season [1,4]. Since activation of sperm motility is unfavourable, especially during short-term storage of sperm, potassium ions are critical components of several sperm extenders or sperm immobilising solutions [21,30]. However, it is unknown whether the presence of potassium ions in an extender is essential for securing live spermatozoa during the cryopreservation process.

The presence of potassium ions in extender is generally justified by the necessity to keep sperm immotile during each step of the freezing/thawing process [25,27]. Early studies on cryopreservation of salmonid fish sperm employed an extender containing 23 mM potassium chloride (KCl), 102 mM sodium chloride (NaCl), 6.2 mM

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calcium chloride ($CaCl_2$) and 0.6 mM magnesium sulphate ($MgSO_4$) buffered with 200 mM Tris-HCl [33,34]. Several other authors used an extender developed by Erdahl and Graham [17] for brown trout (*Salmo trutta*), brook trout (*Salvelinus fontinalis*) and rainbow trout (*Oncorhynchus mykiss*) sperm which consisted of 11 compounds including 34.3 mM KCl. Lahnsteiner et al. [23,24] recommended a universal extender for the cryopreservation of salmonid fish sperm based on 20 mM HEPES buffer supplemented with 40 mM KCl, 103 mM NaCl, 1 mM $CaCl_2$ and 0.8 mM $MgSO_4$. In addition, an extender containing 40 mM KCl was also used by Horváth [19] for cryopreservation of Adriatic grayling *(Thymallus thymallus)*, brown trout and marble trout *(Salmo marmoratus)* sperm. The concentrations of K^+ in these extenders are within the range established in the seminal plasma of salmonids (20–66 mM) [1]. It should be stressed, however, that extenders without potassium ions have also been successfully used for the cryopreservation of salmonid semen [5,10,18]. This raises a question regarding the usefulness of potassium ions for cryopreservation of salmonid semen. To our knowledge, this issue has never been examined experimentally.

Recently, an effective procedure for the cryopreservation of semen from salmonid fish species such as rainbow trout, sex-reversed rainbow trout females, brown trout, brook trout, grayling and European huchen (*Hucho hucho* L.) [11,15,31–33] was developed, using a simple extender (GM) containing only 0.18 M glucose in 9% methanol. This cryopreservation technique secures a very high percentage of post-thaw motile sperm and high fertilisation rates. In addition, the GM extender (which does not contain potassium ions) can be used as a sperm maturation medium for sex-reversed female rainbow trout sperm [12].

The sperm from semen frozen using the GM extender is of good quality (40–70% post-thaw sperm motility [11,15,16,31–33]); however, this is still lower than that of fresh semen. For this reason, the development of an extender that can increase this efficacy is still highly desirable. At present, it remains unknown whether the addition of potassium ions to the GM extender can increase its efficiency, as well as prolong the post-thaw storage period of cryopreserved semen. Such knowledge could lead to improvements in the cryopreservation procedure for salmonid sperm. Moreover, such knowledge is required to better understand the effects of potassium on the physiology of frozen/thawed sperm during the cryopreservation process.

The objective of this study was to determine the effect of the addition of KCl to the GM extender on sperm motility in five species of salmonid fish (rainbow trout, sex-reversed female rainbow trout, whitefish (*Coregonus lavaretus*), brown trout and brook trout). We hypothesised that the addition of potassium ions would have either positive or neutral effects on post-thaw sperm motility parameters. Furthermore, we examined the effect of the presence of potassium ions in the extender on post-thaw storage time and sperm motility parameters.

2. Materials and methods

2.1. Collection of milt and measurement of sperm concentration and seminal plasma osmolality

The experiments were carried out in December (2015) during the natural spawning period on sexually mature males of five species from the *Salmonidae* fish family (rainbow trout of autumn spawning (n = 6), sex-reversed female rainbow trout (n = 6), whitefish (n = 18), brown trout (n = 6) and brook trout (n = 7)) which were born and raised in the Inland Fisheries Institute in Olsztyn, Department of Salmonid Fish Research in Rutki (Poland). Fish were housed in 3 m^3 concrete ponds supplied with water from the Radunia River, with oxygen saturation levels maintained at 85–95% and temperatures of 3–6 °C during spawning. Before milt collection, males were anesthetised using 1 ppm Propiscin (IFI, Żabieniec, Poland). Milt was obtained by gentle abdominal massage, with special care to avoid blood, urine, or faecal contamination, and collected individually in an open glass beaker (0.5 cm depth). Samples with visible contamination were discarded. In the case of sex-reversed female rainbow trout, masculinisation using 11 β-hydroxyandrostenedione was performed by following the protocol described by Kuźmiński and Dobosz [22]. Milt was obtained post-mortem by cutting the testes and gently squeezing through double-layer gauze to remove any testicular tissue. Sperm concentrations were measured using the spectrophotometric method [9]. The osmotic pressure of seminal plasma was measured using a Minitüb Abfüll-u Labortechnik apparatus (Tiefenbach, Germany). Approval was given by the Animal Experiments Committee in Olsztyn, Poland.

2.2. Effects of cryopreservation on sperm motility parameters

2.2.1. Cryopreservation protocol

Cryopreservation followed the previously described procedure using GM extender and 0.25 ml straws [11,15,31–33]. Semen was loaded into 0.25 ml plastic straws, placed on a 3 cm high frame and equilibrated for 15 min on ice. Subsequently, the straws were floated on the surface of liquid nitrogen for 5 min and then immersed in liquid nitrogen. The straws were then thawed by immersion in a water bath at 40 °C for 5 s. Six straws were cryopreserved for each experimental variant: two straws were thawed for testing the effect of potassium ion concentrations, two straws were used for testing the effect of post-thaw storage and the remaining two straws were kept to allow additional experiments/replicates to be conducted. Sperm motility was measured for fresh, equilibrated and frozen/thawed semen.

2.2.2. Effect of potassium ion concentration on sperm motility parameters of fresh, equilibrated and frozen/thawed semen

Semen samples were diluted 1:5 (for brown trout, brook trout, rainbow trout and whitefish) or 1:9 (for sex-reversed rainbow trout) in extender containing 0.18 M glucose in 9% methanol supplemented with 0 (control), 20 or 40 mM KCl. The samples were then processed as outlined in Section 2.2.1.

2.2.3. Comparison of the effects of glucose and potassium ions on sperm motility parameters of fresh, equilibrated and frozen/thawed semen from whitefish

Semen samples were diluted 1:5 in extender containing either 0.18 M glucose, 0.22 M glucose or 0.18 M glucose with 20 mM KCl in 9% methanol. The samples were then processed as outlined in Section 2.2.1. The extender containing 0.22 M glucose was used to evaluate whether a 40 mOsm increase in the osmolality of the extender could compensate the 40 mOsm increase caused by the addition of 20 mM KCl to the extender containing 0.18 M glucose (i.e. both the extender containing 0.18 M glucose +20 mM KCl and the extender containing 0.22 M glucose without KCl have the same osmolality).

2.3. Effect of post-thaw storage on sperm motility

Semen samples were cryopreserved as described in Section 2.2.1. After thawing, semen samples were stored for 30, 60, 120 min (for rainbow trout, sex-reversed rainbow trout and whitefish) and 240 min (for brown trout and brook trout) at 4 °C. After thawing and post-thaw storage at 4 °C, sperm motility parameters were measured for each sample by Computer-Assisted Sperm Analysis (CASA; Hobson Vision Ltd, Baslow, UK; Section 2.4).