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The influence of inhibition of acid phosphatase, β-*N*-acetylglucosaminidase and lactate dehydrogenase present in the sperm of ide (*Leuciscus idus*) on the percentage of fertilised eggs

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ABSTRACT

This study investigated how the inhibition of certain enzymes present in ide sperm influences sperm motility and the percentage of fertilised eggs. The enzymes studied were acid phosphatase (AcP), β-*N*-acetylglucosaminidase (β-NAGase) and lactate dehydrogenase (LDH). None of the inhibitors affected ide sperm motility parameters. The addition of gossypol (a LDH inhibitor) caused a considerable increase in the percentage of fertilised eggs (92–95% compared to 63% in the control). The inhibition of AcP caused a considerable decrease in fertility rate – at the highest inhibitor dose, the percentage of fertilised eggs decreased to 26%. A similar effect was seen after the addition of acetamide (a β-NAGase inhibitor), but in this case the highest dose caused complete inhibition of fertilisation. The results presented here indicate the importance of AcP and β-NAGase in the process of ide fertilisation.

1. Introduction

The process of in vitro fertilisation – an important stage of controlled fish reproduction – is determined by multiple factors including the number of spermatozoa per egg, the activating solution and gamete exposure time. Unfortunately, the typical hatchery protocols for cyprinids (for example, fertilisation with the use of the hatchery water or Woynarovich's solution) often yield unsatisfactory or highly variable results, despite satisfactory sperm motility (above 50%) and good quality eggs. The problems may stem from the fact that the mechanism of fertilisation in fish has not yet been completely elucidated (Bobe and Labbé, 2010; Cabrita et al., 2014; Cejko et al., 2015).

Baba et al. (1994) were the first to pinpoint that acrosin is not the only essential enzyme of mammalian sperm in the fertilisation process, as had previously been believed. Considerable data may be found in the literature confirming that other enzymes have equal importance during fertilisation (Zitta et al., 2006). Whilst investigations addressing this issue have been conducted for many species of mammals and birds, little is known about the role of individual enzymes of fish sperm in the fertilisation process. Fish sperm has been demonstrated to contain various enzymes – for example, acid phosphatase (AcP), lactate dehydrogenase (LDH) and β-*N*-acetylglucosaminidase (β-NAGase) (Lahnsteiner et al., 1995; Piros et al., 2002; Cejko et al., 2015). The activities of the first two enzymes are not only associated with the acrosome, and so their presence is expected in spermatozoa of ide whose gametes are devoid of this structure (Jamieson, 1991). However, the activity of β-NAGase in mammals is linked with the structure of the acrosome (Jauhiainen

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& Vanha-Perttula, 1986; Miranda et al., 2000), and so its role in the semen of fish with non-acrosomal sperm remains unclear.

Acid phosphatase is present in mammalian and bird sperm and takes part in processes including capacitation, hyperactivation, the acrosome reaction and the binding of spermatozoa to the zona pellucida (Urner and Sakkas, 2003). These processes do not occur in non-acrosomal fish spermatozoa, but AcP has been detected in their seminal plasma (Lahnsteiner et al., 1998). After the freezing and thawing of Siberian sturgeon sperm, a high amount of AcP can be measured in the sperm supernatant, suggesting that AcP is an enzyme of spermatozoa origin (Piros et al., 2002).

The high activity of β-NAGase may serve as an indicator of semen quality in Acipenseridae fish, because the activity of this enzyme in Siberian sturgeon semen was 2–4 times higher after freezing and thawing, compared to that of fresh seminal plasma (Piros et al., 2002). Our previous work demonstrated the importance of this enzyme during the fertilisation process of Salmonidae and Acipenseridae fish, as inhibition of β-NAGase in the gametes of Salmonidae fish (whose sperm do not contain acrosomes) caused almost complete inhibition of fertilisation (1% compared to 79% in the control sample). Furthermore, a significant decrease in fertilisation rate from 84% to 12% was reported as a result of β-NAGase inhibition in Siberian sturgeon (Sarosiek et al., 2014), Acipenseridae fish whose spermatozoa do contain acrosomes (Psenicka et al., 2007). The above data therefore suggest that the role of β-NAGase in the fertilisation process may not necessarily be associated with the acrosome reaction.

Lactate dehydrogenase activity has been detected in both non-acrosomal fish sperm such as yellow perch (Ciereszko and Dabrowski et al., 2000), and in Siberian sturgeon sperm, which possess an acrosomal structure (Piros et al., 2002). We observed an increase in enzyme activity in Siberian sturgeon sperm after the freezing and thawing process. Furthermore, a negative correlation between LDH activity in rainbow trout seminal plasma and the percentage of achieved offspring was observed (Lahnsteiner et al., 1998). Therefore, this enzyme may be considered along with AcP and β-NAGase as a useful marker of seminal quality.

This study aims to elucidate the effect of inhibition of AcP, β-NAGase and LDHd on the fertilisation process in cyprinid fish, using ide as a high-value model species. This important group of rheophilic cyprinids has commercial significance for open water managers (millions of hatchery-reared larvae and juveniles are restocked every year to support natural recruitment), and the ornamental fish market due to the presence of colourful forms (Kucharczyk et al., 2008; Sarosiek et al., 2012).

2. Material and methods

2.1. Determination of the effect of enzyme inhibitors on sperm motility

We used the computer-assisted semen analysis (CASA) system to determine the effect of enzyme inhibitors on sperm motility parameters: curvilinear velocity of sperm (VCL; μm s^{-1}), straight-line velocity of sperm (VSL; μm s^{-1}), percentage of motile sperm (MOT; %) and percentage of progressive sperm (PRG; %). Motility analysis was conducted using CRISMAS software (Image House CRISMAS Company Ltd.). Images of sperm motility were recorded with an Olympus BX51 microscope integrated with a Basler 202 K digital camera. To determine sperm motility parameters, semen collected from 4 ide males was mixed (1:1 v/v) with an immobilising medium (TLP buffer: 0.292 g NaCl, 0.012 g KCl, 0.011 g $CaCl_2$, 0.004 g $MgCl_2$, 0.105 g $NaHCO_3$, 0.002 g NaH_2PO_4, 50 mL, pH 8.6 Bavister, 1989), with the addition of 100 mM trehalose (Kowalski et al., 2010) and either: 0.25, 1 or 4 mM ammonium molybdate (AcP inhibitor, Wysocki and Strzeżek, 2006); 0.03, 0.125 or 0.5 M acetamide (β-NAGase inhibitor, Sarosiek et al., 2014); or 0.00625, 0.025 or 0.1 μM gossypol (LDH inhibitor, Ciereszko and Dabrowski, 2000). Semen mixed with immobilising medium without inhibitors was used as a control sample. Semen samples were incubated with the appropriate media for up to 30 min at 4 °C before CASA analysis. Diluted semen was activated prior to fertilisation with an activating medium composed of 0.3% urea, 0.4% NaCl and 0.5% albumin (Woynarovich and Woynarovich, 1980).

2.2. Effect of inhibitors on enzymatic activities

Ammonium molybdate, acetamide and gossypol concentrations were determined from a preliminary experiment, in which their effects on the respective enzymatic activities in the seminal plasma and sperm extract of ide were examined. Spermatozoa were separated from seminal plasma by centrifugation of the semen (8000 × *g*, 10 min). The pellet of spermatozoa was incubated in 20 mM Tris HCl, pH adjusted to 7.6 for 1 h at room temperature. The seminal plasma and sperm extract were used to investigate the effect of inhibitors on the respective enzyme's activity at: 0.25, 0.5, 1.0, 2.0, 4.0 mM ammonium molybdate; 0.0625, 0.125, 0.25, 0.5, 1.0 M acetamide; and 0.00625, 0.0125, 0.025, 0.05, 0.1 μM gossypol (final concentrations). Ammonium molybdate and acetamide were dissolved in ddH_2O, and then added to the seminal plasma. Gossypol was first diluted in ethanol – drop by drop, in the smallest possible volume – and then in ddH_2O. Seminal plasma without inhibitors was used as control sample. Enzymatic activity was expressed as the percentage of reference activity (activity of the untreated samples, defined as 100%).

The activity of AcP was assayed according to the method described by Glogowski et al. (1996), using 5 mM p-nitrophenyl phosphate (disodium salt) in 20 mM citrate buffer, pH 5.0. The activity of β-NAGase was determined as described by according to Jauhiainen and Vanha-Perttula (1986) using 0.5 mM p-nitrophenyl *N*-acetyl-β-*N*-glucosaminide as a substrate in 0.1 M citrate buffer, pH 5.0. That of LDH was measured using a UV-method with pyruvate and NADH (Vassault, 1983).

2.3. Determination of the influence of enzyme inhibitors on ide fertilisation

In the next experiment, enzyme inhibitors (in the concentrations provided above) were added to the activating solution that was used for fertilisation. Eggs were obtained from controlled fertilisation of ides cultured in earth ponds at a fish farm 'Knieja', near