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Carp transferrin can protect spermatozoa against toxic effects of cadmium ions

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ABSTRACT

Cadmium is a widespread heavy metal that enters the aquatic environment and affects many processes involved in fish reproduction such as sperm motility. Fish seminal plasma proteins can protect spermatozoa against toxic effects of heavy metals. The objective of this study was to demonstrate the ability of a major carp seminal plasma protein-transferrin (TF) to bind cadmium ions and to neutralize the toxic effect of cadmium on carp sperm motility. To obtain a high quantity of carp seminal plasma TF necessary for the experiment, immunoaffinity chromatography as a one-step isolation procedure was established. The titration of TF with cadmium ions spectrophotometrically at 247 nm revealed that TF binds cadmium ions at only one spectrophotometrically-sensitive binding site, which suggests that TF is capable of neutralizing the cadmium toxic effect. Indeed, the addition of carp TF to carp semen incubated with 50 ppm cadmium for 48 h led to about a four-times higher percentage of sperm motility ($30.3 \pm 1.1\%$) in comparison to samples incubated with only 50 ppm cadmium ($8.2 \pm 5.2\%$). Similarly, higher values of other parameters of sperm movement measured by a computer-assisted sperm motility analysis system (VSL, VCL and ALH) were observed at the presence of transferrin. In conclusion, our study provides the first evidence that transferrin from carp seminal plasma can protect sperm motility from cadmium toxicity.

1. Introduction

Cadmium (Cd, atomic number 48; relative atomic mass 112.40) is a toxic ubiquitous environmental pollutant. It is released into aquatic environments from industrial sources involved in mining, ore refining and plating processes as well as from natural sources such as rocks and soils (Choi et al., 2007). Cadmium that enters aquatic environments accumulates within the bodies of aquatic organisms. The growth, osmoregulation and reproduction of fish are affected by exposure to cadmium (Kim et al., 2004). Cadmium obstructs numerous reproductive processes in fish such as sexual maturation, spermatogenesis, fertilization success and development of the embryonic and postembryonic stages (Jezierska and Witeska, 2001).

It is well documented that cadmium affects the motility of fish sperm. Besides lowering the percentage of sperm motility, cadmium impacts sperm speed and trajectory of movement (Chyb et al., 2001; Kime et al., 1996, 2001; Rurangwa et al., 1998; Dietrich et al., 2010a,b). These changes can be monitored by the computer-assisted sperm analysis system (CASA), which measures fast and objectively the effect of heavy metal toxicity on fish sperm quality (Lahnsteiner et al., 1998, 2004; Kime et al., 1996; Rurangwa et al., 2001; Abascal et al., 2007). The negative action of cadmium on sperm could be due to displaced essential zinc and calcium (Ebrahimi et al., 1996). Since calcium is a necessary element for sperm movement, the displacement of calcium ions by cadmium may cause the inhibition of sperm motility in many species.

Our studies indicated that transferrin (TF) is a major protein from carp seminal plasma and this protein can be related to sperm motility (Wojtczak et al., 2005, 2007). Recently, we found that carp seminal plasma transferrin has a unique structure and is similar or identical to blood TF (Wojtczak et al., 2007; Dietrich et al., 2010a,b). The presence of transferrin in seminal plasma is a common feature of cyprinids (Dietrich et al., 2010a,b) but its presence in seminal plasma of other fish species has not yet been confirmed directly. Transferrin is recognized as a multi-tasking protein and as a component of a non-specific humoral defense mechanism may play a role in the protection of spermatozoa against bacteria (Stafford et al., 2001; Stafford and Belosevic, 2003; Jurecka et al., 2009). Moreover, the role of TF in protection against oxidative damage was postulated (Saleh and Agarwal, 2002). It is suggested that, besides the binding of iron, transferrin is also a chelator of other metals, including cadmium, for biological defense (Aisen and Listowsky, 1980).

De Smet et al. (2001) indicated that cadmium can bind to carp serum TF. Since TF is present in high amounts in carp seminal plasma and is similar or the same as blood TF (Wojtczak et al., 2005; Dietrich et al., 2010a,b), it can be postulated that seminal TF potentially plays a role in the protection of the carp reproductive tract from cadmium toxicity such as the inhibition of sperm motility. Therefore, the objectives of this study were to determine if carp seminal plasma TF is able to bind cadmium and if the addition of exogenous seminal plasma

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TF neutralizes the toxic effect of cadmium on carp sperm motility. Additionally, a new protocol for one-step isolation of high quantity of carp seminal plasma TF was established.

2. Material and methods

2.1. Source of semen

For the isolation of carp seminal plasma transferrin, milt was collected from 5 to 6 year old European common carp (*Cyprinus carpio* L., n = 10) with an average body mass of 5–7 kg maintained under ambient temperature in the ponds of the Institute of Ichthyobiology and Aquaculture at the Polish Academy of Sciences in Gołysz, Poland. Before the collection of sperm, the fish were transferred from the ponds to tanks with a water temperature of 20 °C reached after seven days. For experiments concerning sperm motility, 2 year old carp with an average body mass (BW) of 1.1 kg were obtained from the Gosławice commercial hatchery in Konin, Poland during the reproductive season. They were held at the University of Warmia and Mazury in a tank with 500 L of circulating aerated water for three days under natural photoperiods at 18 °C for acclimatization.

Approval from the Animal Experiments Committee in Olsztyn, Poland was gained before starting any experiments.

2.2. Sperm sampling

Twenty-four hours before the collection of sperm, common carp were injected intradorsally with Ovopel (one pellet containing of 18–20 μg of GnRH analog and 8–10 mg of metoclopramide per 1 kg of fish BW; Interfish Ltd, Hungary) in a dose of two pellets per kg of fish BW. Milt was stripped by abdominal massage and special care was applied to avoid the contamination of milt. For isolation of transferrin, the milt samples were pooled, and centrifuged at 8000 *g* for 10 min to separate seminal plasma and spermatozoa. The supernatant seminal plasma was centrifuged a second time under the same condition and stored at −80 °C. For experiments concerning sperm motility, milt was collected from individuals and stored on ice.

2.3. Immunoaffinity purification of carp seminal plasma transferrin

The purification procedure of carp seminal plasma TF consisted of the following four steps: 1) coupling of TF to NHS-activated HP column, 2) isolation of anti-TF IgG using TF-coupled NHS-activated column, 3) coupling of anti-TF IgG to NHS-activated HP column, and 4) isolation of TF from carp seminal plasma using column with coupled anti-TF IgG.

2.3.1. Coupling of transferrin to NHS-activated HP column

Purified by the method described previously (Dietrich et al., 2010b), carp seminal plasma TF (1 mg) was lyophilized, diluted with the standard coupling buffer (0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3) and applied to NHS-activated HP column (GE Healthcare, Uppsala, Sweden). Any extra active NHS groups were deactivated by sequential washing with buffer A (0.5 M ethanolamine, 0.5 M NaCl, 8.3) and buffer B (0.1 M acetate, 0.5 M NaCl, pH 4.0). Coupled TF was used as a ligand for affinity purification of antibodies against carp TF raised in our laboratory.

2.3.2. Isolation of anti-transferrin IgG using transferrin-coupled NHS-activated column

First, anti-carp seminal plasma transferrin (anti-TF) antibodies (Dietrich et al., 2010b) were applied on 1 mL HiTrap Protein A (HP) affinity column and total IgGs were purified. The protein A purified total IgGs were diluted with 100 mM Tris–HCl, 0.5 M NaCl (pH 7.6) (v:v) and applied to the TF-coupled NHS-activated column. Unbound IgGs were washed using 50 mM Tris–HCl, 0.15 M NaCl, pH 7.6. Bound anti-TF IgGs were eluted with 0.5 M acetic acid pH 3.4, and immediately neutralized by the addition of 1 M Tris–HCl, pH 9.0. The eluted fraction containing anti-TF IgG was coupled to new NHS column.

2.3.3. Coupling of anti-transferrin IgG to NHS-activated HP column

Before coupling, fractions containing anti-TF IgG were dialyzed overnight against 0.02 M $NaHCO_3$ and lyophilized. The lyophilizate was diluted with the coupling buffer 0.2 M $NaHCO_3$, 0.5 M NaCl (pH 8.3) and coupled to 1 mL HiTrap NHS-activated HP column according to the procedure described earlier (Section 2.3.1). This column was used for immunoaffinity purification of TF from carp seminal plasma.

2.3.4. Isolation of transferrin from carp seminal plasma using column with coupled anti-transferrin IgG

Carp seminal plasma applied to the prepared anti-TF-IgG-coupled NHS-activated column was dialyzed against the binding buffer (0.05 M Tris–HCl, 0.15 M NaCl pH 7.6) at 4 °C for 18 h. Samples (3 mL) were then applied to the column equilibrated with the binding buffer and the column was sealed and incubated for 40 min at room temperature to allow ligand binding. Unbound proteins were washed from the column with 7 mL of binding buffer. The bound TF was eluted from the column with 5 mL of elution buffer (0.5 M acetic acid, pH 3), collected into 1 mL fraction and neutralized by the addition of 200 μL of 1 M Tris–HCl, pH 9.0. The purity of TF was tested using PAGE and SDS-PAGE (Laemmli, 1970).

All of the chromatography steps were performed using the FPLC system (AKTApurifier, GE Healthcare) at a flow rate of 0.5 mL min^{-1}.

2.4. Cadmium binding capacity

The immunoaffinity purified carp seminal plasma TF with a molecular mass of 73.6 kDa (1.6 mg mL^{-1}) was stripped of iron by sequential dialysis against 0.1 citrate/acetate (pH 4.3), distilled water, 0.1 M perchloric acid, water and 0.01 M Tris–HCl (pH 8.0; Harris et al., 1974) at 4 °C. Each dialysis step was performed for about 16 h. Just before the titration an aliquot of 0.5 M $NaHCO_3$ was added to give a 5 mM concentration of bicarbonate. For metal binding to transferrin, it is established that an anion must be concomitantly bound (Aisen and Listowsky, 1980). Carbonate (an anion factor) serves as a binding ligand between metal and protein, excluding water from the two coordination sites. Under physiological conditions carbonate (or possibly bicarbonate) occupies the anion binding sites. The occupancy of the anion-binding site is critical in the ability of transferrin to bind metal. Cadmium binding capacity was measured by the sequential addition of 4–40 μL of 248 ppm $CdCl_2$ to carp apo-TF while equal volumes of deionized water were added to the reference quartz cuvette which also contained apo-TF (De Smet et al., 2001). Ten minutes after each addition, the spectrum from 240 to 270 nm was recorded using Beckman DU 800 spectrophotometer (Analytical Instruments LLC, USA). The absorptivity at the maximal absorptivity wavelength (247 nm) was read for the preparation of the titration curve.

2.5. Experiment 1: the effects of time exposure and dose of cadmium on carp sperm motility

A preliminary experiment was performed to evaluate the time of exposure and dose of cadmium which influenced the percentage of sperm motility. The cadmium chloride was diluted in the immobilizing buffer (94 mM NaCl, 27 mM KCl, 50 mM glycine, 15 mM Tris–HCl, pH 7.5). The two-step method for motility measurement described by Rurangwa et al. (2001) was used in the experiment. As a first step, carp sperm (n = 4) was diluted 50-fold in the immobilizing buffer containing the following concentration of cadmium: 10, 50, and 100 ppm or in the control (no cadmium added). After gentle mixing the samples were incubated for 24 and 48 h at 4 °C until sperm motility analysis.