Identification of oxidatively modified proteins due to cryopreservation of carp semen¹

Agnieszka Mostek, Mariola Słowińska, Sylwia Judycka, Halina Karol, Andrzej Ciereszko, and Mariola A. Dietrich²

Department of Gamete and Embryo Biology, Institute of Animal Reproduction and Food Research, Polish Academy of Sciences in Olsztyn, Tuwima 10, 10-748 Olsztyn, Poland

ABSTRACT: During semen cryopreservation, spermatozoa are exposed to physical and chemical stressors that result in their functional and structural damage. Growing evidence suggests that most cryoinjuries result from oxidative stress accompanying sperm cryopreservation. Elevated amounts of reactive oxygen species (ROS) generated during cryopreservation can react with sperm macromolecules, including proteins. The goal of this study was to investigate the oxidative modifications (measured as carbonylation level changes) of carp spermatozoa proteins triggered by the cryopreservation process. Flow cytometry and computer-assisted sperm analysis were used to evaluate changes in viability, ROS level, and motility of spermatozoa. The spermatozoa proteins that were specifically carbonylated were identified and quantified by Western blotting, in conjunction with 2-dimensional electrophoresis (2D-oxyblot) and matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometry. Cryopreservation decreased spermatozoa motility ($P < 0.01$) and viability ($P <$

0.0001) and significantly increased ($P < 0.0001$) the number of ROS-positive cells. We identified 25 protein spots, corresponding to 19 proteins, with increases ($P < 0.05$) in carbonylation level due to freezing/thawing. The identified proteins are involved in motility, metabolism, calcium-ion binding, signal transduction, protein folding, and intracellular transport. The results suggest that carbonylation of flagellar proteins can result in motility disorders and may contribute to the reduced percentage of motile spermatozoa and disturbances in movement trajectory after sperm cryopreservation. Moreover, cryopreservation may contribute to impaired cellular respiration, ATP regeneration, disturbances of Ca^{2+} turnover, unfolding of cytoplasmic or histone proteins, disturbances of cell signaling and intracellular transport, and reduced membrane stability. Our results contribute to the knowledge concerning cryoinjury and to further development of a modified cryopreservation procedure aimed at minimizing oxidative damage of carp sperm proteins.

Key words: carbonylation, carp, cryopreservation, oxidation, proteome, semen

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²Corresponding author: m.dietrich@pan.olsztyn.pl

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INTRODUCTION

Cryopreservation of fish sperm provides a tool to optimize reproduction and increase larval production, thereby improving breeding and fish conservation programs. However, it is well established that cryopreservation causes osmotic changes and oxidative damage that have sublethal or lethal effects on vertebrate spermatozoa (Ball, 2008)

resulting in reduced semen quality, including that of carp (Li et al., 2010a). Proteomic studies revealed cryopreservation-induced changes in spermatozoa protein profiles (Li et al., 2010b) and disturbances of plasma membrane permeability leading to leakage of sperm proteins into the extracellular medium (Dietrich et al., 2015).

The exact etiology of cryoinjury is not fully elucidated; however, increasing evidence suggests that the root of most injuries stems from oxidative stress (Bogle et al., 2017). Increased levels of reactive oxygen species (ROS) generated during cryopreservation can react with sperm DNA, lipids, and proteins, causing damage to cell structures and multiple disturbances of cell physiology (Agarwal et al., 2014). Our recent studies have indicated that cryopreservation of bull semen triggers the oxidation of sperm proteins involved in capacitation (Mostek et al., 2017) and may lead to cryocapacitation. Although capacitation does not occur in fishes, Li et al. (2010a) showed that cryopreservation induced ROS generation and oxidation of total carp sperm proteins. However, the proteins that are oxidized during cryopreservation and the resultant physiological consequences are unknown.

The aim of this study was to investigate changes in protein oxidation levels between fresh, fresh-diluted, and cryopreserved carp semen. Given that protein carbonylation is a hallmark of oxidative damage (Fedorova et al., 2014), a 2,4-dinitrophenylhydrazine (DNPH)-based approach was used to determine the levels of protein carbonyl groups. We identified, for the first time, specific proteins that were carbonylated during the cryopreservation of carp semen.

MATERIALS AND METHODS

Semen Collection

Semen was collected from common carp (*Cyprinus carpio*) males maintained at the Institute of Ichthyobiology and Aquaculture of the Polish Academy of Sciences in Gołysz, Poland. Healthy-looking males were randomly selected from a larger population of spawners on the basis of external signs of maturity during the natural spawning period. The group of 5- to 7-yr-old carp with an average body weight of 5 to 7 kg was placed in a 2.5-m³ tank in aerated water at 19 to 20 °C and held for a 2-day adaptation period. Twenty-four hours before the collection of carp semen, the males were injected intradorsally with Ovopel (one pellet containing 18 to 20 µg of a GnRH analogue

and 8 to 10 mg of metoclopramide per 1 kg of fish body weight; Interfish Ltd., Hungary). The milt was obtained in the middle of the spawning season (31 May) by gentle abdominal massage, taking care not to pollute them with urine, feces, or blood. Approval from the Animal Experiments Committee in Olsztyn, Poland (no. 93/2011) was received prior to experimentation.

The quality of the fresh carp semen was briefly evaluated to select 6 males that fulfilled the minimum quality standard for sperm cryopreservation (80% sperm motility and a sperm concentration of at least 10×10^9 sperm/mL).

Cryopreservation of Semen

A freezing protocol recommended for carp semen cryopreservation described by Kopeika (1986) and further modified by Li et al. (2010b) was applied. Semen samples ($n = 6$ for each experimental group) were diluted (1:9) in an extender consisting of 59 mM NaCl, 6.3 mM KCl, 0.68 mM CaCl₂, 2.1 mM Mg₂SO₄, 27 mM NaHCO₃, 3.4 mM sucrose, 69 mM D-mannitol, 118 mM Tris-HCl, pH 8.1, and 16% ethylene glycol. After dilution with the extender, 1 part of the sample was taken for future analysis as fresh diluted and the second part was immediately packed into 0.5-mL straws and held in liquid nitrogen vapour 3 cm over the surface of the liquid nitrogen for 5 min. Following freezing, the straws were placed directly into liquid nitrogen for storage. The straws were subsequently thawed by immersing them in a water bath (40 °C) for 13 s. The thawing procedure was performed in duplicate. Thawed sperm samples were immediately used for the analysis of sperm motility parameters and flow cytometry. The fresh-diluted samples were stored at room temperature and analyzed within 5 min after dilution with extender. Sperm concentration of fresh-diluted semen ranged from 17 to 21×10^9 sperm/mL.

Analysis of Fresh, Fresh-Diluted, and Cryopreserved Semen

The motility parameters of carp spermatozoa from fresh, fresh-diluted, and cryopreserved semen were measured using Computer Assisted Sperm Analysis (CASA) as described by Wojtczak et al. (2007). The 2-step method for motility measurement described by Rurangwa et al. (2002) was used. Sperm motility parameters were analyzed with a 15- to 30-s post-activation time using a Hobson Sperm Tracker (Hobson Vision Ltd., Baslow UK) with the setting described earlier by Wojtczak et al.

(2007). Each sample was analyzed in duplicate. For further analysis, percentage of motile sperm (MOT) straight line velocity (VSL), curvilinear velocity (VCL), amplitude of lateral head displacement (ALH), average path velocity (VAP), and linearity (LIN) were chosen. The sperm concentration was measured by spectrophotometry (Ciereszko and Dabrowski, 1993).

Flow Cytometry Analyses of Sperm Viability and Oxidative Stress

The flow cytometry analyses of fresh, fresh-diluted, and cryopreserved carp semen were performed using the Muse Cell Analyzer (EMD Millipore, Billerica, USA) as described by Mostek et al. (2017). Viability and spermatozoa quantification were assessed using Muse Count and Viability Reagent (EMD Millipore) according to the manufacturer's protocol. Semen was diluted 5,000 times in PBS for fresh samples and 500 times for fresh-diluted and cryopreserved semen samples to obtain concentrations in the range of 1×10^5 to 1×10^7 sperm/mL. Subsequently, 20 μ L of each semen suspension was mixed with 380 μ L of Muse Count and Viability Reagent and incubated for 5 min at room temperature.

The ROS levels of carp spermatozoa were measured using a Muse Oxidative Stress Kit (EMD Millipore) that simultaneously determines the count and percentage of cells undergoing oxidative stress based on the intracellular detection of superoxide radicals. The procedure was performed in accordance with the manufacturer's recommendations. Fresh semen was diluted 5,000 times in 1X Assay Buffer, whereas fresh-diluted and cryopreserved semen was diluted 500 times to obtain concentrations in the range of 1×10^6 to 1×10^7 sperm/mL. Subsequently, 10 μ L of the semen suspensions was mixed with 190 μ L of Muse Oxidative Stress working solution and incubated at 37 °C for 30 min. To accurately determine the dividing point between ROS⁺ and ROS⁻ populations, a positive control setup was applied, containing completely oxidized sperm cells. The positive control was performed by incubating the sperm cells in the presence of 3% H₂O₂, a superoxide anion generator, for 1 h at room temperature. All measurements were conducted in triplicate.

Detection of Oxidized Proteins With Oxyblots

Sperm extracts of fresh, fresh-diluted, and cryopreserved semen were prepared as previously

described in Dietrich et al. (2015). Fresh, fresh-diluted and cryopreserved sperm protein extracts ($n = 6$ in each group), containing 150 μ g of proteins, were derivatized with a 4-fold volume of DNPH solution or derivatization-control solution (Oxyblot Protein Oxidation Detection Kit, EMD Millipore). The protein samples were purified with a Clean-Up Kit (GE Healthcare, Uppsala, Sweden) according to the manufacturer's protocol. The final pellet was dissolved in rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 2% [v/v] IPG buffer 3–10 NL, 50 mM DTT, and bromophenol blue). Insoluble components were removed by centrifugation at $10,000 \times g$ for 5 min. Protein concentration was measured with the Bradford protein assay (Coomassie Plus Assay Reagent, Thermo Fisher Scientific).

Samples containing 80 μ g of protein were loaded onto 7-cm Immobiline DryStrips, at non-linear pH range 3 to 10 (GE Healthcare), and rehydrated for 12 h. Proteins were separated by isoelectric focusing and SDS-PAGE using parameters described in Mostek et al. (2017). The control gels treated earlier with derivatization-control solution were stained with colloidal Coomassie Brilliant Blue (CBB) G-250 and scanned at 300 dpi using an Image-Scanner III (GE Healthcare).

The gels with DNPH-derivatized proteins were transferred to a polyvinylidene fluoride membrane (15 V/gel for 2 h) and blocked for 1 h with 1% BSA in PBS-T buffer (PBS with 0.5% [v/v] Tween-20). Oxyblots were incubated with primary rabbit anti-DNP antibody (1:150) overnight at 4 °C (Oxyblot Protein Oxidation Detection Kit, EMD Millipore). Membranes were washed 3 times in PBS-T buffer and incubated for 1 h with an anti-rabbit IgG HRP-conjugated secondary antibody diluted to a 1:300 ratio (Oxyblot Protein Oxidation Detection Kit, EMD Millipore). The membranes were washed 3 times in PBS-T buffer and developed with an Opti-4CN Substrate Kit (Bio-Rad, Hercules, USA) for 15 min. Membranes were dried and scanned with Molecular Imager VersaDoc (Bio-Rad).

Analysis of 2-Dimensional Gel Images and Oxyblots

SameSpots software (Totalab, Newcastle, UK) was used to match and analyze protein spots among different gels and blots corresponding to fresh, fresh-diluted, and cryopreserved semen samples. Normalized spot intensities on oxyblots were compared between treatments. Protein spots that

showed changes ($P < 0.05$) in carbonylation level on oxyblots were subsequently matched with corresponding proteins on gels in order to verify if there were quantitative changes at the protein level. The spots that showed changes only on oxyblots and did not show intensity changes on gel replicates were considered as spots with altered carbonylation levels.

The spots showing differences in protein carbonylation between fresh, fresh-diluted, and cryopreserved semen samples were manually cut out from the gel and subjected to digestion and protein identification using matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometry (MALDI-TOF/TOF).

Protein Digestion and MALDI TOF/TOF Protein Identification

Gel plugs were washed and destained as described earlier (Mostek et al., 2017). The proteins were digested overnight using a trypsin solution (0.2 $\mu\text{g}/\mu\text{L}$ trypsin in 20 mM ammonium bicarbonate) (Promega, Madison, USA). Peptide samples were analyzed on the Autoflex-ToF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). The MS together with MS/MS spectra were searched using the Mascot Server (Matrix Science, London, UK) of the National Center for Biotechnology Information *Bony fishes* database. Search parameters were as follows: trypsin cleavage, monoisotopic masses MH^+ , one missed cleavage site allowed, carbamidomethylation of cysteine as the fixed modification, oxidation of methionine as the variable modification, fragment ion mass tolerance of 0.5 Da, and parent ion mass tolerance of 100 ppm. Matches considered statistically significant ($P < 0.05$) by Mascot with at least 2 correctly identified parent ions were regarded as correct hits. Proteins identified as predicted/uncharacterized were searched in terms of protein sequence similarity using the Basic Local Alignment Search Tool (BLAST).

Gene Ontology Analysis

Gene ontology (GO) annotation was used to determine the function and cellular localization of proteins in our dataset. The GI numbers of identified proteins that showed differences in protein carbonylation were matched to the UniProtKB database (www.uniprot.org) to obtain GO annotation using the categories “molecular function” and “cellular component.”

Statistical Analysis

The statistical analyses of fresh, fresh-diluted, and cryopreserved semen were made using one-way ANOVA and post hoc Tukey's test at a significance level of 0.05 using GraphPad Prism software (GraphPad Software Inc., San Diego, USA). The results are presented as means $\pm$ SD. For statistical procedures, data percentages were transformed by arcsin square-root transformations. Prior to statistical analyses, the data were tested for normality with the Kolmogorov–Smirnov test.

Normalized spot intensities on oxyblots were compared between treatments using one-way ANOVA at a significance level of 0.05, with SameSpots software. Protein spot intensities were normalized to calibrate data between different sample runs. Moreover, normalization corrected the experimental variation of samples (for example, differences in sample loading) by a unique gain factor for each sample—a scalar multiple that was applied to each feature abundance measurement.

RESULTS

Sperm Motility Parameters

Dilution of carp semen with extender decreased the motility parameters including VCL, VSL, VAP, and LIN ($P < 0.05$) but did not affect the percentage of sperm motility and ALH. On the other hand, cryopreservation decreased the percentage of sperm motility and all measured motility parameters (ALH, VCL, VSL, VAP, LIN) ($P < 0.01$) (Figure 1).

Sperm Viability and ROS Measurements

Dilution did not significantly change the viability nor the number of ROS-positive cells of carp semen. On the other hand, cryopreservation was associated with a 40.2% reduction in viability ($P < 0.0001$) (Figure 2A) and dramatically (11-fold) increased the number of ROS-positive cells (Figure 2B) ($P < 0.0001$). Representative plots and histograms displaying changes in viability and ROS-positive cell contents in fresh, fresh-diluted, and cryopreserved semen are shown in Figure 3.

Identification of Carbonylated Proteins

We detected 403 matched protein spots on gel replicates and 191 matched carbonylated spots on oxyblot replicates ($n = 5$). We identified 25 spots, corresponding to 19 proteins, which showed a

Figure 1. Effect of carp sperm dilution and cryopreservation on sperm motility parameters ($n = 6$ in each group). Motility—percentage of sperm motility, VCL—curvilinear velocity, VSL—straight line velocity, VAP—average path velocity, ALH—amplitude of lateral head displacement, LIN—linearity. Results are expressed as means + SD. Different letters indicate significant differences between parameters of fresh, fresh-diluted, and cryopreserved semen ($P < 0.05$).

significant increase in carbonylation level after dilution and/or cryopreservation (Table 1, Figure 4, and Supplementary Figure S1). Three of the identified proteins occurred in 2 to 4 proteoforms: testis-specific gene 10 protein (spots 814, 825, 902, and 918) cilia- and flagella-associated protein 161 (spots

435, 451, and 811) and radial spoke head protein 4 homolog (spots 996 and 1,015).

Three protein spots (756, 737, and 988) showed increased carbonylation levels due to dilution (Figure 4, Supplementary Figure S2B, see online supplementary material for a colour version of

Figure 2. The effect of sperm dilution and cryopreservation on viability (A) and ROS-positive cell content (B) of carp spermatozoa ($n = 6$ in each group). Results are expressed as means + SD. Different letters indicate significant differences between parameters of fresh, fresh-diluted and cryopreserved semen ($P < 0.05$).

Figure 3. Representative histograms displaying live and dead carp spermatozoa populations of fresh (A), fresh-diluted (B), and cryopreserved semen (C). Representative histograms showing ROS-positive and ROS-negative carp spermatozoa populations of fresh (D), fresh-diluted (E), and cryopreserved semen (F).

this figure), and the remaining 22 spots showed increased carbonylation due to cryopreservation (Figure 4, Supplementary Figure S2A, see online supplementary material for a colour version of this figure). The protein spots showing increased carbonylation levels detected on oxyblots did not exhibit significant changes at the protein level on gel replicates. Figure 5 shows the alignment between a representative 2D-gel and oxyblot.

Principal component analysis distinguished the differences in the abundance of 25 protein spots in all three groups: fresh, fresh-diluted, and cryopreserved spermatozoa proteins (Figure 6). The first principal component accounted for 55.81% of the total variance suggesting that the greatest differences in protein carbonylation occurred between cryopreserved semen and the 2 remaining groups—fresh and fresh-diluted semen.

GO Analysis of Carbonylated Proteins

Functional analysis showed that the majority of carbonylated proteins were involved in sperm motility (8 proteins) and metabolism (4 proteins). The additional proteins were assigned to the following functional groups: signal transduction (2 proteins), chaperone (2 proteins), intracellular transport and fusion of plasma membranes (2 proteins), and

calcium ion binding (1 protein). The majority of proteins were related to flagella (12 spots) and cytoplasm (11 spots) (Table 1).

DISCUSSION

The results of the experiments described in this study demonstrate that cryopreservation affects the motility of carp spermatozoa, and alterations in movement trajectories were already observed during dilution with extender. Moreover, cryopreservation of carp semen was associated with a significant increase in ROS production. These results are the first to demonstrate the impact of dilution and cryopreservation of carp semen on carbonylation levels of individual sperm proteins. Three protein spots showed increased carbonylation levels due to dilution, whereas 22 spots showed increased carbonylation due to freezing/thawing. The majority of carbonylated proteins are structural and motor proteins of sperm flagella that are involved in sperm motility and metabolism.

It is well known that the cryopreservation process results in a decrease of carp sperm motility parameters (Li et al., 2010a, 2013; Dietrich et al., 2015; Horokhovatskyi et al., 2016) and viability (Watson, 2000; Li et al., 2010a). Our results indicate that the motility parameters including VCL,

Table 1. Proteins exhibiting significant change in protein oxidation ($P < 0.05$) after dilution or/and cryopreservation

Spot no in Figure 4	Protein name	GI number	Sequence coverage (%)	Protein score	Ion score >30	Function in sperm cells	Cellular localization
Spots showing increased carbonylation level due to dilution							
756	PREDICTED: cilia- and flagella-associated protein 206	gi 221271948	31	313	3	Sperm motility	Sperm flagella
737	PREDICTED: enoyl-CoA hydratase, mitochondrial-like	gi 119119	30	153	2	Metabolism	Mitochondria
988	PREDICTED: malate dehydrogenase, cytoplasmic-like isoform X2	gi 82082933	34	236	4	Metabolism	Cytoplasm
Spots showing increased carbonylation level due to cryopreservation							
1015	Hypothetical protein cypCar_00030249 (BLAST: Radial spoke head protein 4 homolog; cover 94%)	gi 966643197	33	448	5	Sperm motility	Sperm flagella
996	Radial spoke head protein 4 homolog	gi 60415990	37	318	3	Sperm motility	Sperm flagella
773	Predicted: LRP2-binding protein	gi 1101593470	27	239	4	Intracellular transport, fusion of plasma membranes	Cytoplasm
817	Predicted: α -soluble NSF attachment protein isoform X1	gi 1101593048	67	368	2	Intracellular transport, fusion of plasma membranes	Cytoplasm
803	PREDICTED: EF-hand calcium-binding domain-containing protein 1-like	gi 1101650860	72	264	4	Calcium ion binding	Cytoplasm
902	PREDICTED: testis-specific gene 10 protein	gi 81885028	58	585	4	Sperm motility	Sperm flagella
918	PREDICTED: testis-specific gene 10 protein	gi 81885028	38	386	4	Sperm motility	Sperm flagella
814	PREDICTED: testis-specific gene 10 protein	gi 81885028	31	300	2	Sperm motility	Sperm flagella
825	PREDICTED: testis-specific gene 10 protein	gi 81885028	56	441	3	Sperm motility	Sperm flagella
299	PREDICTED: histone-binding protein RBBP7 isoform X1	gi 82241288	33	467	5	Chaperone	Nucleus
632	PREDICTED: dynein light chain 1, axonemal	gi 82235945	38	345	5	Sperm motility	Sperm flagella
435	Predicted: cilia- and flagella-associated protein 161	gi 1101628625	19	227	3	Sperm motility	Sperm flagella
811	PREDICTED: cilia- and flagella-associated protein 161-like	gi 82178197	28	402	4	Sperm motility	Sperm flagella
451	Hypothetical protein cypCar_00007971 (BLAST: Cilia- and flagella-associated protein 161; cover 100%)	gi 966657857	42	326	4	Sperm motility	Sperm flagella
796	PREDICTED: protein phosphatase 1B-like	gi 548444	17	109	2	Signal transduction	Cytoplasm
849	PREDICTED: cAMP-dependent protein kinase type I- α regulatory subunit-like	gi 75571446	30	371	5	Sperm motility	Sperm flagella
856	heat shock cognate 70 kDa, partial	gi 2495341	33	412	4	Chaperone	Cytoplasm
858	β -actin, partial	gi 67462093	52	342	5	Sperm motility	Cytoplasm
873	PREDICTED: 14-3-3 protein β/α -A	gi 82592598	48	306	2	Signal transduction	Cytoplasm
884	PREDICTED: transaldolase-like protein	gi 6648092	31	287	3	Metabolism	Cytoplasm
968	PREDICTED: proteasome subunit beta type-6	gi 108860907	46	323	4	Metabolism	Cytoplasm
977	PREDICTED: creatine kinase B-type	gi 125293	23	194	2	Sperm motility	Cytoplasm

VSL, VAP, and LIN were already affected by effector dilution. On the other hand, dilution of carp semen did not affect the percentage of sperm motility, which is consistent with the results of previous

studies (Li et al., 2010a). Although a cryoprotectant protects cells against freezing damage provoked by intracellular ice crystal formation and excessive dehydration, it may also cause detrimental effects on

Figure 4. Representative oxyblots of fresh (A), fresh-diluted (B), and cryopreserved carp semen (C). Differentially carbonylated protein spots are numbered and listed in Table 1. Protein spots with increased carbonylation levels due to semen dilution with extender are marked in blue circles, and spots that showed increased carbonylation due to cryopreservation are marked in red circles.

sperm physiology, due to osmotic shock or direct biochemical injury (Watson, 1995; Viveiros et al., 2012). Although the ethylene glycol-based extenders are considered the most optimal for carp cryopreservation (Li et al., 2010a), our results suggest that a decrease in several motility parameters (so far attributed to freezing/thawing of the semen) may already occur during the dilution step.

Our results confirmed previous reports that the cryopreservation of carp semen causes a dramatic increase in ROS production (Li et al., 2010a). The

possible reason for a rapid generation of ROS during the freezing/thawing process is electron leakage from damaged sperm mitochondria (Sabeur and Ball, 2006). Furthermore, Li et al. (2010a) showed that generation of ROS, accompanying carp sperm cryopreservation, triggers carbonylation of total proteins. Consistently, we identified specific carbonylated proteins during the cryopreservation process of carp semen. Since carbonylation can potentially lead to damage to the structure of sperm proteins (Fedorova et al., 2014), the identification of

Figure 5. Alignment of representative 2-dimensional gel and oxyblot of cryopreserved semen. Pink color displays protein spots on the gel, and green color indicates carbonylated spots on an oxyblot. Images of 2-dimensional gels and oxyblots were aligned and analyzed with SameSpots software (Totalabs).

individual carbonylated proteins is important for a better understanding of the mechanisms leading to cryopreservation-induced disturbances of sperm function.

It is unknown at present whether the observed oxidative stress affects both dead and live cell populations, as our results were based on the MUSE Cell Analyzer which operates using nonmodifiable reagent-linked protocols. Therefore, further studies should focus on the simultaneous measurement of the number of membrane intact cells and superoxide production with 2 types of fluorescent staining (for example, SYTOXh Green and DHE or propidium iodide and BODIPY used for zebrafish sperm, [Hagedorn et al., 2012](#)) in order to investigate

the occurrence of oxidative stress both in live and dead cell populations.

Our results showed that cryopreservation was associated with the oxidation of flagellar proteins associated with sperm motility. The most distinct carbonylation was found for 2 proteins, identified as testis gene 10 specific proteins and cilia- and flagella-associated proteins (CFAPs) that were carbonylated in multiple proteoforms and variants. Testis gene 10 specific proteins, which were found to be carbonylated in 4 proteoforms, are structural proteins of the sperm tail fibrous sheath-structure surrounding the axoneme and outer dense fibers ([Modarressi et al., 2004](#)). In turn, CFAPs known also as sperm coiled-coil domain containing proteins, are present in sperm in multiple variants, belonging to the dynein regulatory complex ([Merveille et al., 2011](#)). Our study showed that carbonylation of different forms of this protein occurs during subsequent cryopreservation steps. Dilution of semen with extender was associated with the increased carbonylation of CFAP 206, whereas freezing/thawing was associated with the carbonylation of three proteoforms of CFAP 161. These results suggest that the movement apparatus of carp spermatozoa is differentially affected by dilution and freezing/thawing. Moreover, we recently reported the carbonylation of CFAP 161 during the cryopreservation of bull semen ([Mostek et al., 2017](#)). These results suggest that CFAPs may be especially susceptible to oxidative damage in both mammalian and fish sperm.

The majority of proteins that were carbonylated during cryopreservation were axoneme-bound

Figure 6. PCA score plot obtained for carp spermatozoa proteins showing alterations in carbonylation during cryopreservation. Replicates ($n = 6$ in each group) of different semen samples are represented as follows: pink circles—fresh semen, blue circles—fresh-diluted semen, purple circles—cryopreserved semen.

proteins such as radial spoke head proteins, dynein light chain, cAMP-dependent protein kinase, proteasome subunit β , and β -actin. Radial spoke head proteins play essential roles in regulating flagellar bending by modulation of dynein-driven microtubule sliding (Gingras et al., 1998; Inaba and Mizuno, 2016). Our previous results revealed that sperm motility of carp is associated with proteasomes and β -actin (Dietrich et al., 2016). Moreover, sperm motility in salmonid fish is additionally regulated by cAMP-dependent protein kinase (Itoh et al., 2003). Therefore, carbonylation of the above axonemal proteins during freezing/thawing may have detrimental effects on sperm motility caused by flagellar bending disorders in cryopreserved spermatozoa (Inaba, 2011).

Our results showed carbonylation of several enzymes involved in energetic metabolism, such as malate dehydrogenase, enoyl-CoA hydratase, and creatine kinase B-type. It is known that basal ATP production in fish occurs by oxidative phosphorylation, which requires NADH and FADH₂ molecules produced during glycolysis and the Krebs cycle. Since glycolytic and Krebs cycle enzymes, such as Enoyl-CoA hydratase and malate dehydrogenase, were already carbonylated by the stage of semen dilution, it is possible that the addition of cryoprotectant may interfere with the production of acetyl coenzyme A or the conversion of oxaloacetate to malate in the Krebs cycle leading to a disruption in sperm bioenergetic processes. Moreover, carbonylation of creatine kinase (as reported in our study) may indicate disturbances in ATP regeneration in carp sperm, which may result in flagella motility disorders. Mitochondrial oxidative phosphorylation does not meet the energetic demand for ATP due to rapid intracellular ATP consumption accompanying the motility phase (Dzyuba et al., 2015). Thus, it is suggested that the major role in the motile spermatozoa bioenergetics is assigned to adenylate- and creatine-phosphate metabolic activity catalyzed by adenylate kinase and creatine kinase, respectively (Cosson, 2010; Dzyuba et al., 2015). Since sperm motility duration in fish is much shorter than that of mammals and differs significantly among fish species, ATP generation may involve various metabolic strategies. Therefore, a more detailed description of the implications of these strategies is needed for a better understanding of metabolic processes in fish spermatozoa.

It is well established that calcium ions are pivotal in the initiation of sperm motility in carp (Dietrich et al., 2011). Previously, we identified a number of calcium-binding proteins in carp

sperm, including parvalbumin, calreticulin, and calmodulin, that may take part in the mobilization of intracellular Ca²⁺ and control of the Ca²⁺ gradient during sperm movement (Dietrich et al., 2011, 2016). In this study, we identified EF-hand calcium-binding domain-containing protein 1-like, which participates in the regulation of calcium levels in sperm cells. The exact function of this protein remains unknown; however, structural EF-hand domains have been reported to play a role in buffering intracellular calcium levels (Lewit-Bentley and Réty, 2000). In summary, carbonylation of proteins involved in regulating Ca²⁺ levels suggests possible disturbances of calcium turnover and initiation of sperm motility in carp.

The cryopreservation process induced the carbonylation of chaperone proteins such as heat shock cognate 70-kDa (HSC70) and histone-binding protein RBBP7. These proteins help us to ensure proper functioning of spermatozoa, which closely depends on maintaining the correct 3-dimensional structure of proteins. HSC70 is also known as Heat Shock Protein A8 (HSPA8), and it is a constitutive member of a highly conserved chaperone family HSP70. In mammals, HSC70 is postulated to enhance sperm survival in vitro (Elliott et al., 2009; Holt et al., 2015). The mechanisms involved in the protection of spermatozoa by HSC70 remain unclear, but are probably related to an increase in plasma membrane fluidity (Moein-Vaziri et al., 2014). Recent studies have shown that addition of external HSC70 stabilizes the bull sperm plasma membrane and improves the survival of spermatozoa after cryopreservation (Alvarez-Rodríguez et al., 2013; Holt et al., 2015). On the other hand, histone chaperones, such as RBBP7, are integral subunits of protein complexes involved in acetylation/deacetylation of histone proteins, and they play an important role in protecting chromatin structure (Murzina et al., 2008). In contrast to higher vertebrates that contain protamines as the dominant chromatin proteins, carp spermatozoa chromatin contains histones (Ausió, 1999). Therefore, it can be suggested that the carbonylation of the above proteins impairs the correct 3-dimensional structure of histones, which is crucial for proper chromatin condensation and preservation of genetic material and may alter plasma membrane fluidity during cryopreservation.

Our results indicated carbonylation of proteins involved in cell signaling, such as protein phosphatase 1B like and 14-3-3 protein β/α -A. Cell signal transduction is mediated by the reversible phosphorylation of structural and regulatory

proteins that is controlled by protein kinases and phosphatases working independently in a balance (Fardilha et al., 2011). In mammals, protein phosphatases are direct players in the regulation of sperm movement (Vijayaraghavan et al., 1996). Moreover, Li et al. (2013) showed that cryopreservation of carp semen is accompanied by changes in the phosphorylation pattern of sperm proteins. Carbonylation of protein phosphatase, recorded in our study, can potentially affect the phosphorylation of sperm proteins observed during the cryopreservation of carp semen. Reversible phosphorylation involves multiple cascades of biochemical events, which require cooperation with other proteins that regulate the function of phosphatases, such as 14-3-3 proteins (Fardilha et al., 2011). Additional studies are necessary to verify whether carbonylation of proteins involved in protein phosphorylation can be associated with altered protein phosphorylation observed after cryopreservation of carp semen.

Cryopreservation of carp semen was associated with the carbonylation of proteins involved in fusion and stability of plasma membranes, such as α -soluble NSF-attachment proteins (α -SNAP) and low density lipoprotein receptor-related protein-2 (LRP2-binding protein). α -SNAPs of carp spermatozoa were found to be affected by the cryopreservation of semen (Li et al., 2010b). In mammalian sperm cells, these proteins participate in membrane fusion and exocytosis by playing a direct role in the acrosome reaction (Tomes et al., 2005). In turn, LRP2-binding protein is known as a receptor for low density lipoprotein (LDL), which is a key component of sperm seminal plasma. LDL is crucial in maintaining proper membrane fluidity and its levels have been reported to fluctuate significantly during the reproduction period (Kocaman et al., 2005). Moreover, it is well known that LDL has a cryoprotective effect and has been widely used as a membrane stabilizing agent of semen extenders in various mammalian species (Iaffaldano et al., 2014). Carbonylation of proteins involved in fusion and stabilization of plasma membranes can result in disturbances of intracellular transport and in lower freezability of carp semen due to reduced membrane stabilization. Moreover, loss of sperm plasma membrane stability can lead to a leakage of the intracellular proteins of spermatozoa into the extracellular medium, which was demonstrated in our previous study (Dietrich et al., 2015).

Some of the carbonylated carp sperm proteins identified in the present study correspond to proteins previously identified as being sensitive to

cryopreservation. Creatine kinase B-type, proteasome subunit β , and HSC70 were reported to be released from spermatozoa into the extracellular medium (Dietrich et al., 2015), whereas α -SNAP (as indicated above) decreased in abundance in sperm cells after freezing/thawing (Li et al., 2010b). This suggests that for some proteins the mechanism of cryoinjuries includes both their release from spermatozoa and their oxidative damage. It should be noted that the above-mentioned proteins are present in multiple proteoforms in carp semen. In future studies, the relationship between the susceptibility of individual proteins to oxidation and their quantitative changes requires verification in order to better understand the mechanism of cryoinjury to the structure of particular proteins.

Since the cryopreservation of carp semen is accompanied by oxidative stress, it is important to counteract the negative effects of free radicals. The negative effects of oxidative stress can be potentially prevented by extender supplementation with antioxidant compounds such as enzymatic antioxidants: glutathione, peroxidase, glutathione reductase, superoxide dismutase and catalase or nonenzymatic antioxidants, also known as low molecular mass antioxidants, including reduced glutathione, urate, ascorbic acid, vitamin E (α -tocopherol), carotenoids (β -carotene), ubiquinones, taurine, and hypotaurine (Martínez-Páramo et al., 2012; Amidi et al., 2016). Our results demonstrate that monitoring ROS and protein carbonylation level is useful for the evaluation of oxidative stress during the cryopreservation of carp semen. Therefore, those methods can be used in further studies aimed at testing the utility of particular antioxidants to improve cryopreservation protocols of carp semen.

Taken together, our results demonstrated that cryopreservation of carp semen triggers oxidative damage to proteins involved in motility, metabolism, calcium-ion binding, signal transduction, protein folding, and intracellular transport. Carbonylation of structural and motor proteins of sperm flagella can result in motility disorders and may account for the decreased percentage of motile spermatozoa and disturbances of movement trajectory after sperm cryopreservation. Our results suggest that cryopreservation may contribute to impaired cellular respiration and ATP regeneration, disturbances of Ca^{2+} -mediated initiation of sperm motility, unfolding of cytoplasmic or histone proteins, disturbances of cell signaling and intracellular transport, and reduced membrane stability. Additional studies are necessary to investigate the

precise molecular mechanism of fish sperm physiology and shed light on possible protein disorders due to cryopreservation-triggered oxidative damage. Our results contribute to the development of a modified cryopreservation procedure aimed at minimizing oxidative damage of carp sperm proteins by supplementation of cryopreservation media with antioxidants.

SUPPLEMENTARY DATA

Supplementary data are available at *Animal Frontiers* online.

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