Effect of cryopreservation on lipid composition and antioxidant enzyme activity of seabass (*Lates calcarifer*) sperm

Klaiwattana P.[1]; Srisook, K.[2*]; Srisook E.[3]; Vuthiphandchai V.[4]; Neamvonk J.[5]

Received: March 2014 Accepted: September 2015

Abstract

Cryopreservation of seabass (*Lates calcarifer*) semen is an approach to increase the quantity and quality of seabass fry in aquaculture. However, cold shock can induce sperm injury leading to structural damage of the plasma membrane and loss of motility. Thus, the effect of cryopreservation on fatty acid composition and antioxidant enzyme activities of seabass sperm was determined. In cryopreserved spermatozoa, the proportion of C16:0 was significantly increased (p=0.05), while the proportion of C23:0, C22:6 n3, C22:5 n6, and C18:1n9t decreased significantly after freeze-thawing compared with that in fresh semen ($p\leq$0.05). The relative content of saturated fatty acids significantly increased (p<0.05) but that of polyunsaturated fatty acids decreased (p=0.05) in cryopreserved spermatozoa. Consequently, the ratio of unsaturated/saturated fatty acid was greatly decreased in frozen sperm (p<0.05). Superoxide dismutase and glutathione peroxidase activities in frozen-thawed sperm increased significantly while activity of catalase did not differ at p>0.05. Malondialdehyde levels increased after cryopreservation compared to fresh semen (p=0.05). These results indicated that an increase in superoxide dismutase and glutathione peroxidase activities in cryopreserved spermatozoa did not efficiently scavenge reactive oxygen species leading to an increase in lipid peroxidation, a reduction in polyunsaturated fatty acids and finally irreversible loss of sperm motility.

Keywords: *Lates calcarifer*, Sperm, Cryopreservation, Fatty acid composition, Antioxidant enzymes

1-Biological Science Program and Centre of Excellence for Innovation in Chemistry, Faculty of Science, Burapha University, Chonburi, Thailand.

2-Department of Biochemistry and Centre of Excellence for Innovation in Chemistry, Faculty of Science, Burapha University, Chonburi, Thailand.

3-Department of Chemistry and Centre of Excellence for Innovation in Chemistry, Faculty of Science, Burapha University, Chonburi, Thailand.

4-Department of Aquatic Science, Faculty of Science, Burapha University, Chonburi, Thailand.

5-Department of Mathematics, Faculty of Science, Burapha University, Chonburi, Thailand.

* Corresponding author's e-mail: klaokwan@buu.ac.th

Introduction

Seabass (*Latescal carifer*) is an economically important food fish which is commercially cultured in Thailand. A major problem in their propagation which uses sperm cryopreservation is the low quantity and quality of gametes (Martinez-Paramo *et al*., 2012) and embryos. Freezing sperm can induce cellular injury, structural damage to plasma membranes, reduced metabolic activity, and an irreversible loss in motility as well as reduced viability and fertilization capacity of sperm (Labbe and Maisse, 1996; Bailey *et al.*, 2000; Miller *et al*., 2004). This is commonly referred to as cold shock (Waterhouse *et al*., 2006) and has been associated with disruption and damage to the sperm membrane. The major constituent of the sperm membrane is lipid (Lenzi *et al*., 1996), which is responsible for the fluidity of membrane bilayer (Sanocka and Kurpisz, 2004). Membrane damage may be manifested by the change in its lipid composition. Several studies have reported lipid modification due to cryopreservation (Cerolini *et al*., 2001; Chakrabarty *et al*., 2007; Zaniboni and Cerolini, 2009).

During cryopreservation, semen is exposed to cold shock and atmospheric oxygen, which in turn increases their susceptibility to lipid peroxidation due to higher production of reactive oxygen species (ROS) (Nair *et al*., 2006; Chen *et al*., 2010; Lahnsteiner *et al*., 2010). Spermatozoa are highly susceptible to oxidative stress and particularly to lipid peroxidation due to the high content of polyunsaturated fatty acids in plasma membrane. Lipid peroxidation of sperm membrane destroys the lipid matrix structure, due to attacks from ROS. These attacks ultimately impair sperm function, including sperm motility, functional membrane integrity and fertility, leakage of intracellular enzymes and damage to the sperm DNA (Aitken *et al*., 1993; Baumber *et al*., 2000).

Enzymatic antioxidant defense mechanisms in seminal plasma and spermatozoa include superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) which is described as the defense functioning mechanism against lipid peroxidation maintaining sperm motility and viability (Gadea *et al*., 2004; Lahnsteiner *et al*., 2010; Martinez-Paramo *et al.*, 2012; Shaliutina-Kolesova *et al.*, 2013). Antioxidant enzyme activities (SOD and CAT) are reduced during freezing and thawing in red seabream (*Pagrus major*) sperm (Chen *et al.*, 2010).On the contrary, chilling of seminal plasma and sperm of brown trout (*Salmo trutta fario*) caused no significant difference of these enzymatic activities between 0 and 48 h incubation (Lahnsteiner *et al.*, 2010). Quantification of GPx and SOD activities of European seabass (*Dicentrarchus labrax*) sperm was similar in fresh and cryopreserved samples (Martinez-Paramo *et al.*, 2012). However, there are no available references relative to changes in

biochemical composition in post-thaw sperm of seabass (*L. calcarifer*). The present study focused on studies of fatty acid composition and antioxidant enzyme activities of fresh and cryopreserved seabass sperm.

Materials and methods

Fish

Male broodstock of seabass (*L. calarrifer*) of about 3.2±0.3 kg were obtained from a private fish farm in Bangphakong River region, Bangphakong district, Chachoengsao Province, Thailand in April 2010. The fish were maintained in indoor tanks supplied with sea water at the Department of Aquatic Science, Faculty of Science, Burapha University.

Sperm collection and freezing and thawing procedure

Semen samples were pooled from 6 males and placed on a dry clean petri dish on ice. Sperm motility and sperm density of fresh semen was determined under microscope using the method of Vuthiphandchai *et al*. (2009)**.** Briefly**,** sperm motility was evaluated by activating the sperm sample with 0.8% (w/v) NaCl at room temperature. Percentage of sperm motility was determined within 15 sec after activation. Analyses were repeated three times for each treatment. Pooled semen was divided into 2 groups including fresh semen and cryopreserved semen, each investigated in triplicate. Fresh semen was diluted at a volume ratio 1:1 with Ringer's solution (0.13 mMNaCl, 2.7 mMKCl, 1.4 mM $CaCl_2$, 2.4 mM $NaHCO_3$ and 28 mM glucose, pH 7.9). Aliquot of 0.5 mL of diluted semen was mixed with 0.5 mL of 20% (v/v) dimethyl sulfoxide. After 10 min of equilibration to cryoprotectant, 0.25 mL of semen solution was transferred into a 2.0-mL cryopreservation vial and placed inside the programmable controlled rate freezer (Model 3000, Cryologic Pty., Australia) using software CryoGenesisTM V4.02 for window). The vials were plunged in liquid nitrogen (-196°C) for 24h. Cryovials were removed in liquid nitrogen and thawed in a water bath at 70°C for 30 sec to estimate post-thaw motility of sperm and biochemical composition**.**

Extraction of total lipid and analyses of fatty acid composition

Fresh and thawed semen were centrifuged at 3000 g for 20 min at 4°C to collect spermatozoa. An equal amount of 1.25×10^{10} spermatozoa/ mL was used for lipid analysis. Total lipids were extracted according to the method of Folch *et al.* (1957) with minor modification. The cell pellets (spermatozoa) were washed with PBS and centrifuged at 3000 g for 10 min at 4°C. Total lipids were extracted in chlorofrom: methanol (2:1; v/v) containing 0.002% (w/v) butylated hydroxyl toluene (BHT). The samples were sonicated on ice for 15 min using a Vibra cell ultrasonicator. Thereafter, chloroform: water (1:1; v/v) was added to each sample which was centrifuged at 3000 g for 10 min at 4 °C. The lower chloroform layer was transferred to a

new 15-mL tube and methanol: water (10:9; v/v) was added. Samples were mixed and centrifuged at 3000 g for 10 min at 4°C. The lower chloroform layer was evaporated to dryness under nitrogen and stored at -80°C under nitrogen atmosphere until further analysis. Total lipids were resolved into triacylglycerol, cholesterol and phospholipid classes by column chromatography on silicic acid according the method described by Chakrabarty *et al.* (2007). A glass column (2.5 cm × 40 cm) was packed with slurry of about 6 g of silicic acid (70-230 mesh, Merck) in dichloromethane and washed three times with dichloromethane. Total lipids were redissolved in dichloromethane and applied to the column. The triacylglycerol, cholesterol and phospholipid fractions were collected in 15-mL glass tubes, 15 mL in each. After collection, samples in each tube were separated by thin layer chromatography (TLC) to identify lipid class. Lipids in each class were pooled and dried by rotary evaporator**.** Finally, lipid fractions were stored at -80°C under nitrogen atmosphere until further analysis.

Fatty acids from phospholipid fractions were transmethylated as described by Morrison and Smith (1964). Phospholipid from each sample and nonadecanoic acid (C19:0, Supelco Sigma-Aldrich, U.S.A.) as an internal standard were resuspended in 1 mL of 14% (v/v) boron trifluoride in methanol under nitrogen in a new tube. Tubes were closed with the screw cap, vortexed and incubated in 95°C water bath for 10 min under nitrogen to protect lipid peroxidation before cooling in tap water. The methyl ester was extracted by adding 1 mL of hexane and 0.5 mL of distilled water, shaking vigorously and centrifuging at 3000 g for 10 min at 4 °C. Finally, the upper hexane layer was transferred into a new tube and analyzed for fatty acid methyl esters by gas chromatography (GC**;** HP 6890, Agilent Technologies , U.S.A.), using a polyethylene glycol capillary column (DB-Wax; length 30 m × 320 µm I.D. × 0.25 µm film thickness, Agilent Technologies, U.S.A.) with a flame ionization detector (FID). Injection volume 2 µL was done in split injection mode. The initial oven temperature was 100°C; after a 5 min delay, temperature was increased at 5°C /min to 150°C which was maintained for 5 min. Oven temperature was then increased to 180°C at 1.5°C/min and, again kept constant for 5 min after which it was increased at the same rate to 210°C for 35 min. Total run time was 100 min. Peak values were authenticated by comparing their retention times to that obtained by analysis of a standard mixture of fatty acid methyl ester (FAMEs, Supelco Sigma-Aldrich, U.S.A.**)** and docosapentaenoic acid methyl ester (DPA**,** NU-CHEK PREP, U.S.A**.**) under the same conditions. The percentage of each individual fatty acid in a sample was calculated by comparison of the total detected fatty acid peak value area to that of a known concentration of non adecanate methyl ester (internal

standard) using methods described by Yimyuan (2007).

Assays of antioxidant enzyme activity

A sperm pellet was resuspended with 1.5 mL of Ca-F HBSS and centrifuged at 3000 g for 20 minutes at 4 °C. One mL of Ca-F HBSS was added to the cell pellet, and sonicated on ice for 10 min with a Vibra cell ultrasonicator**.** The mixture was then centrifuged at 3000 g for 20 minutes at 4°C and the supernatant transferred to a microtube and stored at -80°C until further analysis. The antioxidant enzyme study included determination of activities of GPx, SOD and CAT. Protein content in supernatant was determined using Quick Start Bradford Dye Reagent following manufacturer's instruction (Biorad, U.S.A).

GPx activity assay was carried out after Li *et al*. (2000) with some modifications. Ten µL of supernatant was added to the reaction mixture containing 50 mM potassium phosphate buffer pH 7.8, 0.1 M EDTA and 0.1 M NaN_3, 50 mM glutathione (GSH), glutathione reductase (2.4U/mL) and 1.5 mM NADPH that was incubated at 25°C for 10 min. The reaction was induced by the addition of 1.5 mM tert-butyl hydroperoxide and determined at 340 nm for 3 min at 1 sec intervals after initiation using UV-Visible spectrophotometer (HP, Germany). One unit of GPx activity is defined as that amount of protein that oxidized 1 µm NADPH per minutes.

Assay of SOD activity was performed according to the method described by Ukada *et al*. (1997) with partial modification. Ten µL of sample was incubated in the reaction mixture consisting of 50 mM sodium carbonate buffer pH 9.4, 3 mM xanthine, 3 mM EDTA and 5 mM XTT. The reaction was initiated by xanthine oxidase (XO) (60 mU/mL) equilibrated at 25°C. The assay was performed in 96 well microtiter plates. Absorbance change was monitored with a microplate reader (Versamax, U.S.A.) at 470 nm for 20 min at 30 sec intervals by a kinetic program. One unit of SOD activity was defined as the amount of the enzyme causing 50% inhibition of the reduction of XTT.

CAT activity was analyzed according to method of Latchoumycandane *et al.* (2002). Twenty µL of sample was incubated in a reaction mixture consisting of 2.4 mL of 50 mM sodium phosphate buffer pH 7.0 containing 0.1% (v/v) triton X-100.to which was added 30 µL of Ca-F HBSS. The reaction was started by the addition of 10 µL of 19 mM hydrogen peroxide and equilibrated at 25°C. Enzymatic activity was determined by following the decrease in absorbance at 240 nm, during 3 min**,** at 1 sec intervals due to H_2O_2 reduction to H_2O and O_2 in the presence of catalase using a UV-Visible spectrophotometer with a kinetic program. One enzyme unit is defined as the amount of CAT capable of transforming hydrogen peroxide per minute per milligram protein.

Determination of malondialdehyde (MDA) content

The thiobarbituric acid assay was used to assess lipid peroxidation using the method of Niki (2000) with partial modification. A volume of 20 μL of sample and 40 μL of TBA reagent (containing 1.5g trichloroacetic acid, 0.0375g thiobarbituric acid, 2.2 mL of 2 mM butylated hydroxy toluene (BHT) in 70% ethanol, and 2.5 mL of 1M HCl) were mixed into a 1.5-mL microtube and 0.6 μL of 50 mMBHT in 70% ethanol was then added. The solution mixture was incubated for 15 min at 95°C in a boiling water bath and then cooled in ice-cold water for 30 min. After cooling, the test tube was centrifuged at 12,000 g for 20 min at 4°C. The upper layer was transferred into a 96 well-microplate and absorbance recorded at 535 nm with a microplate reader. Assays were performed in triplicate. The calculation was based on a standard curve prepared with different concentrations of MDA (0 – 0.025 μM). Results are expressed in μM of MDA per milligram protein.

Statistical analysis

Results are expressed as means±SD (n=3) and were analyzed by Mann-Whitney U test using SPSS program (Statistical Package for Social Science Version 21.0). A value of $p<0.05$ was considered significant.

Results

Effect of cryopreservation on sperm motility

Average sperm motility of fresh semen before the experiment was 80.0±0.0%. After cryopreservation, motility decreased relative to fresh semen by 55.0±1.4% ($p<0.05$).

Effect of cryopreservation on fatty acid composition

Fatty acid composition of sperm phospholipids contained 16 fatty acids and did not differ between the tested groups (Table 1). The most abundant fatty acid was docosahexaenoic acid (DHA; C22:6n3). Palmitic acid (C16:0) was the major saturate, oleic acid (C18:1n9c), the major monounsaturates, and DHA (C22:6n3), the major polyunsaturates in fresh and cryopreserved sperm. Cryopreserved sperm had lower levels of DHA (C22:6n3 $p=0.050$), docosapentaenoic acid (DPA; C22:5n3, $p=0.034$), elaidic acid (C18:1n9t, $p=0.034$) and tricosanoic acid (C23:0, $p=0.034$) when compared to fresh seabass sperm (Table 1). The effect of cryopreservation on sperm phospholipid was to increase the level of palmitic acid (C16:0, $p=0.050$).

Table 1: Fatty acid composition (percentage of weight) of phospholipid fraction from fresh and cryopreserved sperm.

Fatty acid	Before cryopreservation	After cryopreservation	*p*- value
Saturated fatty acid			
C14:0	0.3 ± 0.1	0.4 ± 0.2	*p*=1.000
C15:0	0.3 ± 0.0	0.3 ± 0.0	*p*=0.456
C16:0	20.6 ± 2.4	25.9 ± 0.6	*p*=0.050
C17:0	0.8 ±0.0	0.7 ± 0.0	*p*=0.456
C18:0	9.4 ± 0.6	9.9 ± 0.8	*p*=0.376
C23:0	0.7 ± 0.0	0.5 ± 0.0	*p*=0.034
Monounsaturated fatty acid			
C16:1	0.8 ± 0.1	0.8 ± 0.1	*p*=1.000
C18:1n9c	13.6 ± 0.7	12.9 ± 0.5	*p*=0.275
C18:1n9t	3.1 ± 0.1	2.7 ± 0.0	*p*=0.034
C20:1	0.5 ± 0.0	0.5 ± 0.1	*p*=0.480
C24:1 n9	0.3 ± 0.0	0.3 ± 0.0	*p*=0.317
Polyunsaturated fatty acid			
C18:2n6c	0.5 ± 0.0	0.4 ± 0.0	*p*=0.456
C20:4n6	8.4 ± 0.3	7.9 ± 0.2	*p*=0.184
C20:5n3(EPA)	2.4 ± 0.1	2.2 ± 0.1	*p*=0.068
C22:5n6 (DPA)	2.2 ± 0.0	1.9 ± 0.0	*p*=0.034
C22:6n3 (DHA)	36.2 ± 0.7	32.6 ± 1.1	*p*=0.050
∑SAT	32.0 ±1.8	37.7 ± 1.6	*p*=0.046
∑MUFA	18.3 ± 0.7	17.2 ± 0.4	*p*=0.127
∑PUFA	49.7 ± 1.2	45.1 ± 1.3	*p*=0.050
Ratio of ∑Unsat/∑sat	2.1 ± 0.2	1.7 ± 0.1	*p*=0.046

Results are presented as mean ± SD of 3 samples. ∑Unsat /∑sat=∑ of total unsaturated fatty acids/∑ of total saturated fatty acids.

Table 2: **Antioxidant enzyme activities and MDA level in fresh and frozen-thawed sperm.**

Antioxidant enzyme activity	Before cryopreservation	cryopreservation sperm	*p*- value
GPx activity(U/mg protein)	140.0±6.3	153.8±5.3	*p*=0.050
SOD activity(U/mg protein)	14.5±0.2	21.3±1.7	*p*=0.050
CAT activity(U/mg protein)	154.5±5.9	160.7±16.0	*p*=0.827
MDA concentration(µM/mg protein)	67.7±2.8	133.1 ± 8.3	*p*=0.050

Results are expressed as means±SD of 3 samples.

Relative content of saturated fatty acid in cryopreserved sperm increased significantly (p=0.046) in phospholipids while the relative content of polyunsaturated fatty acids, especially DHA (C22:6 n3) and DPA (C22:5 n6), decreased markedly ($p \leq 0.050$). Therefore, there was a significant decrease (p=0.046) in the ratio of total unsaturated/saturated fatty acids between fresh and cryopreserved cells.

Effect of cryopreservation on antioxidant enzyme activities and lipid peroxidation level.

In accord with other fish species, CAT, SOD and GPx activities were found in seabass sperm (Table 2). CAT activity did not differ significantly after

freezing-thawing as compared with fresh sperm. SOD and GPx activities of sperm are significantly (p=0.050) higher for 24h after cryopreservation than in fresh semen.

When seabass semen was cryopreserved for 24h, the concentration of MDA, a biomarker of lipid peroxidation, increased significantly (p=0.050) in sperm. The MDA concentrations were 67.7±3.4 µM/mg protein in fresh sperm and 133.1±8.3 µM/mg protein after cryopreservation.

Discussion

The biochemical basis of seabass sperm is not well understood. Thus, this study investigated the fatty acid composition and content from phospholipid fraction of seabass sperm due to the majority of lipid in vertebrate sperm cells is polar lipid (phospholipid and sterol) (Bell *et al*., 1996; Lenzi *et al.,* 1996). To the best of our knowledge, this is the first fatty acid profile in seabass sperm. These results are similar to those reported in various fish species such as, European seabass (*D. labrax)* (Bell *et al*., 1996) and rainbow trout (*Oncorhynchus mykiss*) (Labbe and Maisse, 1996; Liu *et al.,* 1997). Compared to other fish sperm (Bell *et al*., 1996; Labbe and Maisse, 1996; Liu *et al.,* 1997), the percentage of DHA content in seabass spermatozoa was higher than in European seabass and rainbow trout.

During cryopreservation, the levels of some saturated and unsaturated fatty acid in spermatozoa were changed. The proportions of PUFAs of seabass (*L. calcarifer*) spermatozoa were significantly reduced while the proportion of saturated fatty acid was increased during cryopreservation as compared to fresh spermatozoa. This was similar to that reported in boar (*Sus scrofa*) spermatozoa (Cerolini *et al.,* 2001). Such a decrease in total PUFAs in frozen-thawed spermatozoa might be a consequence of the relative increase in the proportions of saturates or of an actual loss of PUFAs owing to lipid peroxidation (Cerolini *et al.,* 2001). During cryopreservation, semen is exposed to cold shock and atmospheric oxygen, which in turn increases their susceptibility to lipid peroxidation due to high production of ROS (Bucak *et al*., 2008). ROS induced damage to sperm is mediated by the oxidative attack of sperm phospholipid bound PUFAs, resulting in lipid peroxidation and loss in motility. Some authors reported that freezing and thawing of spermatozoa is associated with an increase in ROS generation (Baumber *et al*., 2000; Kankofer *et al.,* 2005).

Spermatozoa and seminal plasma have enzymatic (GPx, SOD, and CAT) and non-enzymatic (α-tocopherol, ascorbic acid, uric acid etc.) defense mechanisms against ROS and lipid peroxidation in semen (Lahnsteiner *et al*., 2010). Enzymatic antioxidant defense mechanism in semen has been described as an important mechanism in maintaining sperm motility and viability (Bucak *et al*., 2008). Limited literature is available on the antioxidant systems of fish sperm. Seminal plasma

and sperm of teleost (burbot- *Lota lota*, perch- *Perca fluviatilis*, bleak- *Alburnus alburnus*, brown trout- *Salmo trutta*, common carp- *Cyprinus carpio*, and brook trout- *Salvelinus fontinalis*) and chondrostean (Russian sturgeon- *Acipenser gueldenstaedtii*, Siberian sturgeon- *Acipenser baerii*, and starlet- *Acipenser ruthenus*) fishes contain different types of antioxidant including SOD, CAT, GPx, glutathione reductase and methionine sulfoxide reductase (Lahnsteiner and Mansour, 2010; Lahnsteiner *et al.*, 2011; Shaliutina-Kolesova *et al.*, 2013). Chen et al. (2010) reported also that SOD and CAT activities were found in sperm of red seabream (*Pagrus major*). In accord with other fish species, seabass sperm contained at antioxidant enzyme activities including SOD, CAT and GPx.

As shown in Table 2, activities of SOD and GPx in seabass sperm were significantly increased while CAT activity did not significantly differ after frozen-thawed as compared to fresh sperm. The MDA concentration of seabass sperm was increased after cryopreservation. Our results are similar to those obtained by Cerolini *et al*. (2001) who observed that cryopreserved sperm had the higher SOD activity. However, these findings are in contrast with those of Chen *et al.* (2010), who reported reduced antioxidant enzyme activities (SOD and CAT) during freezing and thawing in red seabream sperm and increased MDA during long term cryopreservation. On the contrary, Lahnsteiner *et al*. (2011) reported that MDA concentration was not different in fresh and cryopreserved sperm of brook trout (*Salvelinus fontinalis*) and rainbow trout. Recently, activity of GPX and SOD of European seabass was reported not to differ significantly after freezing and thawing as compared to fresh spermatozoa while MDA quantification remained constant (Martinez-Paramo *et al*., 2012). Different effects of cryopreservation might be species-specific and/or related to differences on cryopreservation protocols such as a different extender solution, cryoprotectant, handling time or semen collecting methods (Martinez-Paramo *et al*., 2012; Shaliutina-Kolesova *et al*., 2015). However, this study indicates a need for superoxide anion radical and hydrogen peroxide scavengers. SOD scavenges superoxide anion radical and generates hydrogen peroxide, which is in turn scavenged by GPx and CAT but in this study SOD and GPx activities increased and hydrogen peroxide accumulated. Possibly the increase in SOD GPx activities were insufficient to prevent lipid peroxidation efficiently. This might have caused a reduction in the proportion of polyunsaturated fatty acids and an increased in saturated fatty acids in cryopreserved sperm and ultimately a loss in motility and fertilization. Observations that the addition of antioxidants to semen improves sperm quality provide indirect evidence for the damaging effect of ROS on sperm function. Previous studies have suggested supplementation

with antioxidant molecules to reduce oxidative stress during the sperm storage process and improve the storage quality of chilled and cryopreserved semen (Bucak *et al.*, 2008; Michael *et al*., 2009; Lahnsteiner and Mansour, 2010). Catalase improved sperm motility parameters and membrane integrity in semen of brown trout, burbot and yellow perch but not that of *A. alburnus* (Lahnsteiner and Mansour, 2010) and it is likely useful also for protection of spermatozoa from ROS during storage.

In conclusion, this study demonstrates that cryopreservation caused changes in fatty acid composition and SOD as well as GPx activities in seabass spermatozoa. The increase in such enzyme activity was nsufficient to scavenge ROS and might lead to a significant increase in lipid peroxidation and a corresponding reduction in polyunsaturated fatty acids in cryopreserved sperm. This study provides a new approach to the cryopreservation of seabass sperm, and could contribute to the improvement of its cryopreservation. However, further studies are required in which to examine the effect of supplemental antioxidants or antioxidant enzymes to storage media of seabass semen to improve sperm quality after cryopreservation.

Acknowledgements

The work was financially supported by Burapha University and also by the Centre of Excellence for Innovation in Chemistry (PERCH-CIC), Commission on Higher Education, Ministry of Education, Thailand. Authors are grateful to Prof. Dr. Frederick W.H. Beamish, Faculty of Science, Burapha University, for his comments and English correction. We thank I. Thadsri and P. Onkwanmaung, Department of Aquatic Science, Faculty of Science, Burapha University for technical assistance in animal handling, collection and preservation of seabass semen.

References

Aitken, R.J., Buckingham, D. and Harkiss, D., 1993. Use of a xanthine oxidase free radical generating system to investigate the cytotoxic effects of reactive oxygen species on human spermatozoa. *Journal of Reproduction and Fertility*, 97, 441–445.

Bailey, J.E., Bilodeau, J.F. and Cormier, N., 2000. Semen cryopreservation in domestic animals: A damaging and capacitating phenomenon. *Journal of Andrology*, 21, 1-7.

Baumber, J., Ball, B.A., Gravance, C.G., Medina, V. and Davies-Morel, M.C.G., 2000. The effect of reactive oxygen species on equine sperm motility, viability, acrosomal integrity, mitochondrial membrane potential, and membran lipid peroxidation. *Journal of Andrology*, 21, 895-902.

Bell, M.V., Dick, J.R., Thrush, M. and Navarro, J.C., 1996. Decreased 20:4n−6/20:5n−3 ratio in sperm from cultured sea bass, *Dicentrarchus labrax*, broodstock compared with wild fish. *Aquaculture*, 144, 189-199.

Bucak, M.N., Ateşşahinb, A. and Yücec, A., 2008. Effect of anti-oxidants and oxidative stress parameters on ram semen after the freeze–thawing process. *Theriogenology*, 75, 128-134.

Cerolini, S., Maldjian, A., Pizzi, F. and Gliozzi, T.M., 2001. Changes in sperm quality and lipid coposition during cryopreservation of boar semen. *Reproduction*, 121, 395-401.

Chakrabarty, J., Banerjee, D., Pal, D., De, J., Ghosh, A. and Majumder, G.C., 2007. Shedding off specific lipid constituents from sperm cell membrane during cryopreservation. *Cryobiology*, 54, 27–35.

Chen, Y.K., Liu, Q.H., Li, J., Xiao, Z.Z., Xu, S.H., Shi, X.H. and Ma, D.Y., 2010. Effect of long-term cryopreservation on physiological characteristics, antioxidant activities and lipid peroxidation of red seabream (*Pagrus major*) sperm. *Cryobiology*, 61, 189–193.

Folch, J., Lees, M. and Stanley, S.G.H., 1957. A simple methods for the isolation and purification of total lipids from animal tissue. *Journal of Biology and Chemistry*, 226, 38-45.

Gadea, J., Sellés, E., Marco, M.A., Coy, P., Matás, C., Romar, R. and Ruiz, S., 2004. Decrease in glutathione content in boar sperm after cryopreservation. Effect of the addition of reduced glutathione to the freezing and thawing extenders. *Theriogenology*, 62, 690-701.

Kankofer, M., Kolm, G., Aurich, J. and Auric, C., 2005. Activity of glutathione peroxidase, superoxide dismutase and catalase and lipid peroxidation intensity in stallion semen during storage at 5°C. *Theriogenology*, 63, 1354–1365.

Labbe, C. and Maiss, G., 1996. Influence of rainbow trout thermal acclimation on sperm cryopreservation: relation to change in the lipid composition of the plasma membrane. *Aquaculture*, 145, 281–294.

Lahnsteiner, F. and Mansour, N., 2010. A comparative study on antioxidant systems in semen of species of the Percidae, Salmonidae, Cyprinidae, and Lotidae for improving semen storage techniques. *Aquaculture*, 307, 130-140.

Lahnsteiner, F., Mansour, N. and Plaetzer, K., 2010. Antioxidant systems of brown trout (*Salmotrutta f. fario*) semen. *Animal Reproduction Science*, 119, 314–321.

Lahnsteiner, F., Mansour, N. and Kunz, F.A., 2011. The effect of antioxidants on the quality of cryopreserved semen in two salmonid fish, the brook trout (*Salvelinus fontinalis*) and the rainbow trout (*Oncorhynchus mykiss*). *Theriogenology*, 76, 882–890.

Latchoumycandane, C., Chitra, K.C. and Mathur, P.P., 2002. The effect of methoxychlor on the epididymal antioxidant system of adult rats. *Reproductive Toxicology,* 16, 161–172.

Lenzi, A., Picardo, M., Gandini, L. and Dondero, F., 1996. Lipids of sperm plasma membrane: from polyunsaturated fatty acid considered as markers of sperm function to possible scavenger therapy. *Human Reproduction Update,* 2, 246–256.

Li, S., Yan, T., Yang, J.Q., Oberley, T.D. and Oberley, L.W., 2000. The role of cellular glutathione peroxidase redox regulation in the suppression of tumor cell growth by manganese superoxide dismutase. *Cancer Research,* 60, 3927–3939.

Liu, L., Ciereszko, A., Czesny, S. and Dabrowski, K., 1997. Dietary ascorbylmino phosphate depresses lipid peroxidation in rainbow trout spermatozoa. *Journal of Aquatic Animal Health,* 9, 249-257.

Martinez-Paramo, S., Diogo, P., Dinis, M.T. Herraez, M.P., Sarasquete, C. and Cabrita, E., 2012. Incorporation of ascorbic acid and α-tocopherol to the extender media to enhance antioxidant system of cryopreserved sea bass sperm. *Theriogenology,* 77, 1129-1136.

Michael, A.J., Alexopoulos, C., Pontiki, E.A., Hadjipavlou-Litina, D.J., Saratsis, P., Ververidis, H.N. and Boscos, C.M., 2009. Effect of antioxidant supplementation in semen extenders on semen quality and reactive oxygen species of chilled canine spermatozoa. *Animal Reproduction Science,* 112, 119–135.

Miller, R.R., Sheffera, C.J., Cornetta, C.L., McCleanb, R., MacCallum, C. and Johnstonb, S.D., 2004. Sperm membrane fatty acid composition in the Eastern grey kangaroo (*Macropus giganteus*), koala (*Phascolarctos cinereus*), and common wombat (*Vombatus ursinus*) and its relationship to cold shock injury and cryopreservation success.*Cryobiology,* 49, 137-148.

Morisson, R.W. and Smith, L.W., 2007. Preparation of fatty acid methylesters and dimethylacetals from lipids with boronfluoride-methanol. *Journal of Lipid Research,* 5, 600–608

Nair, S.J., Brar, A.S., Ahuja, C.S., Sangha, S.P. and Chaudhary, K.C., 2006. A comparative study on lipid peroxidation, activities of antioxidant enzymes and viability of cattle and buffalo bull spermatozoa during storage at refrigeration temperature. *Animal Reproduction Science,* 96, 21-29.

Niki, E., 2000. Lipid peroxides .In : Experimental protocols for reactive oxygen and nitrogen species, Taniguchi, N. and Gutteridge, J.M.C. (Eds), pp 156-160, Oxford University Press.

Sanocka, D. and Kurpisz, M., 2004. Reactive oxygen species and sperm cells. *Reproductive Biology and Endocrinology,* 2, 12.

Shaliutina-Kolesova, A., Cosson, J., Lebeda, I., Gazo, I., Shaliutina, O., Dzyuba, B. and Linhart. O., 2015. The influence of cryoprotectants on sturgeon (*Acipenser ruthenus*) sperm quality, DNA integrity, antioxidant responses, and resistance to oxidative stress. *Animal Reproduction Science*, 159, 66–76.

Shaliutina-Kolesova, A., Gazo, I., Cosson, J. and Linhart. O., 2013. Comparison of oxidant and antioxidant status of seminal plasma and spermatozoa of several fish species.*Czech Journal of Animal Science*, 58, 313–320.

Ukeda, H., Maed, S., Ishii, T. and Sawamura, M., 1997. Spectrophotometric assay for superoxide dismutase based on tetrazolium salt 3́-{1-[(Phenylamino) -carbonyl]-3, 4-tetrazolium}-bis(4-methoxy-6-nitro) benzenesulfonic acid hydrate reduction by xanthine–xanthine oxidase. *Analyical Biochemistry*, 251, 206–209.

Vuthiphandchai, V., Chomphuthawa, S. and Nimrat, S., 2009. Cryopreservation of red snapper (*Lutjanus argentimaculatus*) sperm: Effect of cryoprotectants and cooling rates on sperm motility, sperm viability, and fertilization capacity. *Theriogenology*, 72, 129–138.

Waterhouse, K.E., Hofmo, P.O., Tverdal, A. and Miller, R.R., 2006. Within and between breed differences in freezing tolerance and plasma membrane fatty acid composition of boar sperm. *Reproduction Research*, 131, 887-894.

Yimyuan, K., 2007. Diversity and fatty acid composition in thraustochytrids isolated from mangrove leaves at Leam Pak Bia, Phetchaburi Province. Thesis for Master degree,Burapha University, Chonburi, Thailand.

Zaniboni, L. and Cerolini, S., 2009. Liquid storage of turkey semen: Changes in quality parameters, lipid composition and susceptibility to induced in vitro peroxidation in control, n-3 fatty acids and alphatocopherol rich spermatozoa. *Animal Reproduction Science*,112, 51-65.