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Antioxidant defence system in protein fractions of common carp (*Cyprinus carpio*) seminal plasma

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Abstract: There are growing evidences that the damage which is caused to the spermatozoa by the reactive oxygen species (ROS) plays a key role in male infertility. The seminal plasma is endowed with many enzymatic and non-enzymatic antioxidants which protect the spermatozoa against oxidative stress. The present study was undertaken to compare the level of superoxide dismutase (SOD), glutathione reductase (GR) and glutathione peroxidase (GPx) in separated protein fractions of whole common carp *Cyprinus carpio* seminal plasma. Seminal plasma from six male common carp was separated into four fractions. The fractions differed from each other in their total seminal plasma protein content and antioxidant status. The highest value of SOD (2.81 ± 0.11 mU/mg protein) was found in fraction 4, and the lowest (1.83 ± 0.18 mU/mg protein) in fraction 1. There were significant variations (ANOVA; $P = 0.05$) in GR activity, mainly between fractions 1, 2, and 4. The lowest value of GPx activity (14.9 ± 0.4 mU/mg protein) was found in fraction 2; however, the GPx activity showed low variability among fractions. We conclude that distinct seminal plasma fractions differ in antioxidant capacity. The inclusion of fractions with high levels of SOD, GR, and GPx in sperm storage medium may enhance protection against oxidative stress and promote sperm survival during the *in vitro* storage.

Keywords: fish; seminal plasma fractions; proteins; antioxidant activity

Seminal plasma (SP) is a natural medium containing components that provide an optimal environment for the storage of spermatozoa in the sperm duct (Ciereszko et al. 2011). The role of seminal plasma was traditionally assumed to be primarily a medium for sperm transport; however, its isolated constituents demonstrate direct effects on sperm physiology and fertility (Maxwell et al. 2007; Ratto et al. 2011), which differ with species.

The use of specific seminal plasma fractions, rather than the entire supernatant, for preservation of sperm *in vitro* has been recently studied with promising results (Saravia et al. 2007). Barrios et al. (2000) demonstrated by scanning electron

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microscopy that fractions of ram seminal plasma can restore the membrane of spermatozoa damaged by cold shock. The study also showed that *in vitro* incubation of ram sperm in selected fractions of seminal plasma increased the rate of spermatozoon viability. In studies conducted in boar, spermatozoa incubated with SP pre-sperm-rich fraction, sperm-rich fraction, and post-sperm rich fractions, showed the highest percentage motility, with the highest spermatozoon viability recorded after exposure to the sperm-rich fraction (Garcia et al. 2009). Research suggests that seminal plasma fractions show potential as additives for preserving the function of semen; however, their effects have been little studied in fish and need investigation.

Oxidative stress is the result of an imbalance between the production of reactive oxygen species (ROS) and their neutralisation or scavenging by the antioxidant system (Fazeli and Salimi 2016). When the production of ROS by spermatozoon mitochondria is excessive, the gamete's limited defences are rapidly overwhelmed, and oxidative damage can induce lipid peroxidation with a loss of vitality and fertilizing potential (Aitken et al. 1998). Unfortunately, fish spermatozoa possess limited endogenous antioxidant protection and are vulnerable to ROS damage (Koppers et al. 2010; Gazo et al. 2013). Therefore, seminal plasma that is well supplied with an array of antioxidants acting as free radical scavengers can protect the spermatozoa against oxidative stress.

Seminal plasma contains a number of enzymatic antioxidants including superoxide dismutase (SOD), glutathione reductase (GR), catalase (CAT), and glutathione peroxidase (GPx) (Li et al. 2010; Martinez-Paramo et al. 2012). In addition, it possesses a variety of non-enzyme antioxidants that suppress the formation of ROS or counteract their actions (Lahnsteiner et al. 2010; Ciereszko et al. 2011). The antioxidant power of a biological fluid such as seminal plasma can be assessed only through the analysis of individual antioxidants.

The aim of this study was to analyse the antioxidant defence system of common carp *Cyprinus carpio* seminal plasma by measuring SOD, GR, and GPx activity in its separated fractions and to compare the capacity of each fraction to protect spermatozoa from oxidative stress. The common carp was selected, as it is among the most commonly cultured fish species and is a representative model teleost.

MATERIAL AND METHODS

Experimental fish, location and rearing conditions. Procedures were conducted according to the principles of the Ethics Committee for the Protection of Animals in Research of the University of South Bohemia in České Budějovice, Research Institute of Fish Culture and Hydrobiology, Vodňany, based on the EU-harmonised Animal Welfare Act of the Czech Republic.

Male common carp (n = 10) weighing 4.0–4.5 kg were obtained from the experimental station of the Faculty of Fisheries and Protection of Waters, University of South Bohemia at Vodňany, Czech Republic. Prior to experimentation, fish were held in 10 000 l outdoor hatchery tanks with constant flowing pond water. Water parameters followed seasonal ambient conditions in the range 20–22°C, pH 7.0–7.5, and 6.5–7.1 mg O_2/l. Ambient light entered the tanks via netting that prevented fish from escaping.

Semen collection and preparation of seminal plasma. Spermiation was induced with an injection of carp pituitary extract at 1 mg/kg. 24 h post-injection, sperm samples were collected by abdominal massage directly into 10 ml plastic syringes, taking care to prevent contamination with blood, faeces, or urine. Samples were stored 30 min on ice (0–4°C) in closed assay tubes until assessment.

Each sperm sample was initially centrifuged at 700 *g* for 30 min followed by 10 min at 16 000 *g* at 4°C. The seminal plasma was carefully collected and stored at –80°C until analysis. The bicinchoninic acid assay (BCA) with the Infinite M200 photometer (Tecan, Switzerland) were used to determine protein concentrations.

Seminal plasma fractionation. Nanosep® 100 kDa, 50 kDa, 30 kDa, and 10 kDa centrifugal devices (Pall Laboratory, USA) were used to separate the seminal plasma into fractions according to the protein content. The separation of each sample usually started with the 100 kDa molecular weight cutoff (MWCO): 500 µl of SP was centrifuged at low speed, 2000 *g*, for 30 min. The filtrate from the bottom receiver was collected and divided into two aliquots. A 450 µl aliquot was filled to 500 µl with PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4, pH 7.4), briefly mixed, centrifuged in a lower MWCO device, and collected as before. The second SP aliquot (20 µl) containing protein fractions with defined molecular weight (MW) was stored at –80°C until analysis.

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***SDS-gel electrophoresis* (*SDS-PAGE*).** The seminal plasma protein fractions obtained from each male were analysed by 12% polyacrylamide-bisacrylamide gel electrophoresis. Samples were re-suspended in buffer containing 65 mM Tris 10% (v/v) glycerol, 2% (w/v) sodium dodecyl sulphate (SDS), and 5% (v/v) beta-mercaptoethanol and denatured at 95°C for 3 min prior to loading to gels. Protein bands were visualised by Coomassie Brilliant Blue R-250 staining (Applichem, Germany) and scanned. GelQuant v. 2.7.0 (Bio-Imaging Systems, Israel) was applied to determine the MW of protein bands in each fraction.

Evaluation of SOD activity in fractionated seminal plasma. SOD activity was determined by the method of Marklund and Marklund (1974) based on the autoxidation of pyrogallol. Each prepared seminal plasma fraction was centrifuged at 12 000 *g* at 4°C for 30 min. The SOD activity was assessed spectrophotometrically at 420 nm and expressed as the quantity of enzyme per mg of protein. One unit of SOD activity is defined as the amount of the enzyme necessary to produce 50% dismutation of the superoxide radical per min. Results were expressed as international milliunits (mU) per mg of protein and obtained in triplicate for each sample.

Evaluation of GR and GPx activity in fractionated seminal plasma. GR activity was determined spectrophotometrically, measuring NADPH oxidation at 340 nm. GPx activity was assayed based on the rate of NADPH oxidation at 340 nm, by the coupled reaction with GR. The specific activity was determined using the extinction coefficient of 6.22 mM/cm (Lawrence and Burk 1976). One unit of GPx or GR activity was defined as the quantity of the enzyme that consumes 1 µmol of substrate or generates 1 µ*mol* of product per min. The activity was expressed as mU/mg protein. GPx and GR activity was assessed in triplicate for each sample.

Data analysis. Prior to comparison of the variables, data were tested by the Kolmogorov test and the Bartlett test. Values of SOD, GR, and GPx were expressed as mean ± standard deviation (SD) (n =6) and analysed by factorial analysis of variance (ANOVA). Statistical comparison was made by ANOVA followed by Tukey's HSD test for each analysed variable. All analyses were performed at a significance level of $P < 0.05$ using STATISTICA (Version 9.0, 2009) software for MS Windows.

RESULTS

Protein separation by SDS-PAGE and gel imaging. The SDS gel electrophoresis results revealed four distinct fractions with differing total protein content (Figure 1). The molecular weight of separated proteins, based on the band pattern on denatured SDS-PAGE, and band quantity of each fraction are summarised in Table 1. Briefly, the first seminal plasma fraction showed five protein bands with MW in the range 19–73 kDa. Fraction 2 comprised six protein bands of MW 18–57 kDa, fraction 3 consisted of three bands ranging from 35 to 58 kDa, and fraction 4 bands of 17 kDa and 18 kDa (Table 1).

Enzymatic antioxidant activity in seminal plasma fractions. The antioxidant activity was assessed as total SOD, GR, and GPx level.

Superoxide dismutase. The level of SOD varied significantly among seminal plasma fractions (Figure 2A). The highest value of SOD (2.81 ± 0.11 mU/mg protein) was observed in the fraction separated by a Nanosep 10 kDa device (fraction 4), with the lowest (1.83 ± 0.18 mU/mg protein) de-

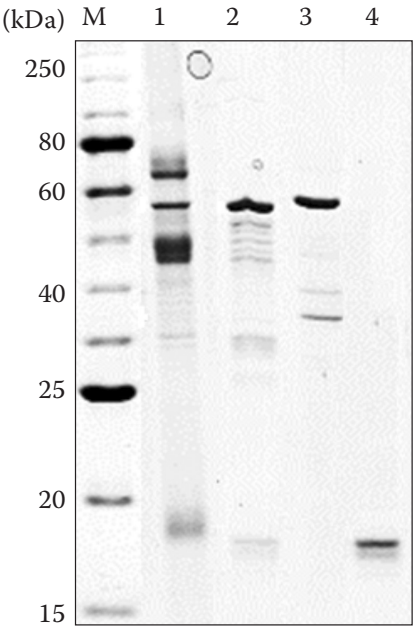

Figure 1. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) pattern of common carp (*Cyprinus carpio*) seminal plasma protein fractions

line 1 = fraction separated in a Nanosep 100 kDa device, line 2 = fraction separated in a Nanosep 50 kDa device, line 3 = fraction separated in a Nanosep 30 kDa device, line 4 = fraction separated in a Nanosep 10 kDa device, M = molecular weight marker

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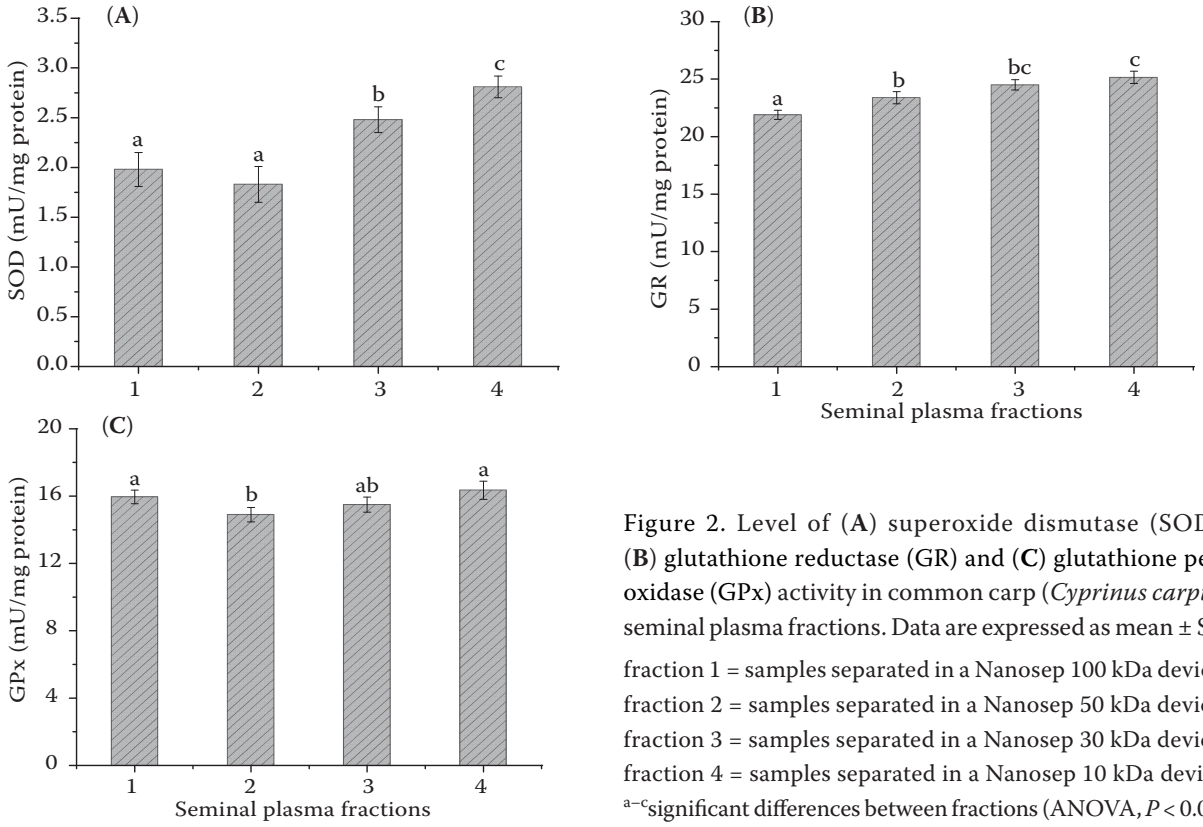

Figure 2. Level of (**A**) superoxide dismutase (SOD), (**B**) glutathione reductase (GR) and (**C**) glutathione peroxidase (GPx) activity in common carp (*Cyprinus carpio*) seminal plasma fractions. Data are expressed as mean ± SD

fraction 1 = samples separated in a Nanosep 100 kDa device, fraction 2 = samples separated in a Nanosep 50 kDa device, fraction 3 = samples separated in a Nanosep 30 kDa device, fraction 4 = samples separated in a Nanosep 10 kDa device

[a–c]significant differences between fractions (ANOVA, $P < 0.05$)

tected in fraction 1 separated by the Nanosep 100 kDa device. There was no significant difference (ANOVA; $P > 0.05$) in SOD activity between the first and the second seminal plasma protein fractions (Figure 2A).

Table 1. Distribution of protein bands in seminal plasma fractions of common carp (*Cyprinus carpio*)

Seminal plasma fractions	Molecular weight of proteins (kDa) (n = 10)
1	73.0 ± 2.67
	57.4 ± 0.64
	49.4 ± 0.79
	31.2 ± 0.44
	19.6 ± 0.54
2	57.5 ± 2.12
	55.0 ± 0.63
	50.3 ± 0.73
	48.2 ± 0.75
	30.5 ± 0.49
	18.3 ± 0.64
3	58.4 ± 2.68
	39.3 ± 0.79
	35.4 ± 1.15
4	18.0 ± 0.60
	17.4 ± 0.43

Glutathione reductase. Significant differences (ANOVA; $P = 0.05$) in GR activity were seen among fractions 1, 2, and 4 separated by Nanosep 100 kDa, 50 kDa, and 10 kDa devices, respectively. The GR activity in seminal plasma protein fractions 2 and 3 ranged from 23.38 ± 0.53 mU/mg protein to 24.5 ± 0.45 mU/mg protein with no significant differences detected between them (Figure 2B). The highest GR level at 25.15 ± 0.54 mU/mg protein was measured in fraction 4.

Glutathione peroxidase. Low variability was observed in GPx activity in seminal plasma protein fractions (Figure 2C) with no significant differences (ANOVA; $P > 0.05$) between fractions 1, 2, and 4. The lowest value of GPx activity (14.9 ± 0.4 mU/mg protein) was found in fraction 2 separated by a Nanosep 50 kDa and the highest (16.35 ± 0.54 mU/mg protein) in fraction 4.

DISCUSSION

Antioxidant activity assessed as the total SOD, GR, and GPx level showed a significant variation

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among selected fractions, confirming the existence of an integrated antioxidant system in individual fractions of common carp seminal plasma. Nevertheless, the highest levels of SOD, GR, and GPx was recorded in fraction 4 that can make this fraction as a candidate to be used for improving storage protocols for sperm.

A large number of seminal plasma components influence the biological quality of semen (Ciereszko et al. 2011; Chacur 2012). Studies have provided direct evidence that specific components can be adsorbed into the surface of spermatozoa and have an important function in the process of capacitation (Manjunath and Therien 2002), sperm transport (Clark 2011), and fertilisation (Barrios et al. 2000). Seminal plasma fractions have also been associated with protective properties and show the ability to restore the integrity of damaged sperm (Barrios et al. 2000; Perez-Pe et al. 2001). In our study, SDS-PAGE electrophoresis was used to define the proteins contained in an SP fraction obtained by centrifugation in a Nanosep device. Four separated fractions were visualised on the gel that correspond to protein bands with the following MW: from 73 to 19 kDa (fraction 1); from 57 to 18 kDa (fraction 2); from 58 to 35 kDa (fraction 3); from 18 to 17 kDa (fraction 4). According to Dietrich et al. (2014, 2017), we can speculate that the protein band identified as 73 kDa belongs to the family of major proteins of carp seminal plasma, transferrin. Proteins of ≥ 50 kDa may be hemopexin-like proteins, whereas proteins with MW ≥ 30 kDa could relate to members of the large group of SP apolipoproteins. Proteins of MW ≤ 19 kDa may be cofilin-2-like proteins (Dietrich et al. 2017). However, during the separation of protein fractions using SDS-PAGE, proteins of similar molecular weight may overlap. Therefore, further investigation of protein content in fractionated seminal plasma is required.

The protective antioxidant system in semen is made up of both enzymes and non-enzymatic factors along with low molecular weight compounds with antioxidant capacity, all of which closely interact to ensure optimum protection against oxidative damage (Walczak-Jedrzejowska et al. 2013). The quantity of enzymes varies among species and is present in both seminal plasma and spermatozoa (Shaliutina-Kolesova et al. 2013). Donkey and stallion semen displays the highest reported seminal plasma antioxidant activity (Cassani et al. 2005), whereas bovine sperm exhibits a low level of activity (Bilodeau et al. 2000). Compared to mammals, information on the mechanism of action of antioxidant enzymatic systems in fish sperm is limited.

SOD is the most abundant enzyme in biological systems (Halliwell 2006) and is considered the first line of defence against the effects of oxyradicals in the cell through catalysing the dismutation of superoxide radicals to hydrogen peroxide and molecular oxygen (Li et al. 2010). We found considerable variation in SOD activity among fractions, with the highest value recorded in fraction 4, indicating that most SOD activity in seminal plasma of common carp is contained in its low molecular weight protein fraction, which may possess beneficial properties. Garcia et al. (2009) separated boar seminal plasma and showed a positive effect of certain fractions on sperm survival during *in vitro* storage. Barrios et al. (2005) reported that ram seminal plasma fraction containing low MW proteins may protect the sperm and reverse spermatozoon damage caused by cold shock. These observations, together with the findings of this study, suggest that separated fractions containing high concentrations of SOD and low MW proteins would be best equipped to counteract an increase in oxygen radical production.

GR provides intracellular defence of sperm against oxidative stress (Cheema et al. 2009), acting against lipid peroxidation (LP) caused by ROS and playing a major role in maintaining sperm quality (Shaliutina-Kolesova et al. 2014). We confirmed GR activity in all separated fractions, contrary to results of Marti et al. (2007) who found GR activity in only two fractions of ram seminal plasma. The variation of GR between fractions in our study showed a trend similar to that of SOD, with the highest value in fraction 4 and the lowest in fraction 1. The source of the low level of GR in fraction 1 may have been the presence of high MW proteins that inhibit the activity of GR.

Differences in GPx activity among fractions were also observed. GPx, a ROS scavenger, exerts a protective effect on spermatozoon membranes from LP damage (Alvarez and Storey 1989) and can inhibit LP of the cell membrane (Sharma and Agarwal 1996). Jervis and Robaire (2001) demonstrated high GPx activity in fractions of ejaculated ram semen, possibly due to increased protein synthesis, and reported a low level of GPx in the

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sperm-rich fraction of ram semen. Three protein bands with peroxidase activity in the sperm-rich fraction of dog semen provided evidence that GPx plays the primary role in the decomposition of hydrogen peroxide (Koziorowska-Gilun and Strzezek 2011). We observed low variation in GPx level in the analysed seminal plasma fractions, hypothetically suggesting similarity in preservation of spermatozoon membrane against ROS. However, GPx activity should not be lower than that of SOD to avoid the production of toxic levels of hydrogen peroxide. GPx and SOD activity in separated fractions should be synergistic. It has been reported that both GPx and SOD play a key role in protecting mammalian sperm against oxygen radical induced damage leading to motility loss (Alvarez and Storey 1989).

CONCLUSION

We confirmed that common carp seminal plasma can be separated by centrifugal devices into four protein fractions. The fractions contained proteins differing in molecular weight as well as in antioxidant activity. The highest levels of SOD, GR, and GPx was recorded in fraction 4 separated by a Nanosep 10 kDa device. We suggest that this fraction can be used as an additive in fish sperm preservation to provide protection to sperm during *in vitro* storage by reducing oxidative stress induced by ROS. Further work is needed to determine whether proteins present in separated seminal plasma fractions can be characterized, synthesized, and used as supplements in sperm preservation media to improve fish fertility.

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