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Trace elements content in semen and their interactions with sperm quality and RedOx status in freshwater fish *Cyprinus carpio*: A correlation study

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ABSTRACT

Objective of the present study was to investigate interactions between trace elements content and RedOx status, as well as sperm quality parameters (motility features, DNA fragmentation) in fish spermatozoa in natural conditions. Reproductively mature male freshwater fish (n = 16) of *Cyprinus carpio* breed were used in the study. Trace elements content was determined in fish milt samples by inductively-coupled plasma optical emission spectrometry (ICP-OES) and by cold-vapor atomic absorption spectroscopy (CV-AAS). Sperm quality evaluation was realized by computer-assisted sperm analysis (CASA) quantifying several parameters: concentration, total motility, progressive motility, distance average path, distance curved line, distance straight line, velocity average path, velocity curved line, velocity straight line, straightness, linearity, amplitude of lateral head displacement and beat cross frequency. The general scheme of descending concentrations of trace metals in semen samples was following: Zn > Fe > Cu > As > Sr > Ni > Mn > Se > Pb > Cr > Cd > Hg. Total motility of spermatozoa was relatively high (91.45%), however progressive motility was not even half of this value (39.47%). Sperm DNA fragmentation values were relatively low (4.00–6.29%). The percentage of immotile spermatozoa showed a significant correlation with all RedOx status parameters and also with DNA fragmentation. Positive statistically significant correlations were observed between trace elements (Mn, Se, Sr, and Zn) and some qualitative spermatozoa parameters (velocity and distance parameters). Cu and Hg content shows similar negative associations with progressive motility. Hg also interacted with production of malondialdehyde. Overall, the present study suggests application of multi-component mixtures of environmentally related trace elements concentrations when assessing the potential reproductive risk.

1. Introduction

In general, it is well known that reproductive system may be considered as barometer of environmental contamination [1]. The main contaminants of environment targeting the male reproductive system are heavy metals [2,51], pesticides [3–6] and endocrine disruptors [7–10].

A number of studies have demonstrated a possible involvement of trace elements in the dys/function of male reproductive system in terms

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of endocrine activity, gametes production and gamete quality, specifically in men [11–14], boar [15], fox [16], buffalo [3], as well as in fish species, namely African catfish [17], Sea bass - *Dicentrarchus labrax* [18], Rainbow trout - *Oncorhynchus mykiss* [19], Banded knifefish - *Gymnotus carapo* [20], and Common carp [21,22]. Other studies demonstrated associations of trace metals with oxidative status parameters, associated with sperm motility parameters in bull *in vivo* [23] and in fish (*Acipenser ruthenus*) in *in vitro* conditions [24]. Several trace metals are considered essential for reproduction [25], however combined higher levels of these elements in seminal plasma may have adverse effect on sperm motility, oxidative stress production and thus may interfere with physiological processes responsible for successful fertilization [26–28]. In terms of bioaccumulation and hard degradability or non-degradability, environmental pollutants such as heavy metals are a serious risk factor for the aquatic organisms. Fish exposed to metals may be affected through a direct effect on the testes resulting in decreased fertility and embryonic development [29].

Bergamo et al. [52] in pilot study described human semen as sensitive biomarker of highly polluted living environment, with the combined measurement of trace elements in association with the overall assessment of semen quality, RedOx parameters and sperm DNA damage. Similar studies in animals, not to mention in fish, are unknown as majority of studies is focused on effect of one metal in high concentrations. This kind of research in fish is insufficient mainly from the perspective of their natural environment contaminated with the multicomponent mixture of toxicants to which they are constantly exposed [30]. Thus, the first objective of the present study was to investigate interactions between trace elements (As, arsenic; Cd, cadmium; Cr, chromium; Cu, cuprum; Fe, iron; Mn, manganese; Ni, nickel; Pb, lead; Se, selenium; Sr, strontium; and Zn, zinc) content and oxidative stress (OS) indices (ROS, reactive oxygen species; TAC, total antioxidant capacity; MDA, malondialdehyde; CP, carbonyl protein), as well as sperm quality parameters (motility and velocity, DNA fragmentation) in fish spermatozoa in natural conditions. The second aim of the study was to evaluate the link between sperm quality (motility and DNA fragmentation) and seminal antioxidant system.

2. Material and methods

2.1. Fish

Reproductively mature male freshwater fish (n = 16) of *Cyprinus carpio* breed were harvested from the experimental pond located at the affiliation of Slovak University of Agriculture in Nitra, University Farm Kolíňany (48°21′14.6″N 18°13′03.2″E) [31]. Fish stocking was realized in March 2015. Catching of the fish was realized from May to June 2015. The freshwater fish common carp (*Cyprinus carpio*) were caught by seine net. In total, 16 male fish were collected. The fish were transferred in polyethylene bags to the laboratory within 20 min for semen collection after catching. Fish were manipulated by a competent person in accordance to the provisions of the national law. After standard ichthyology evaluation (age: 6–9 years; body weight: 1826 ± 295 g; total length: 452 ± 22 mm; standard length: 383 ± 19 mm) semen collection was performed.

2.2. Semen sample collection

Fish were transferred to the laboratory of Department of Poultry Science and Small Animal Husbandry where were humanely sacrificed. Testes were surgically removed *post mortem* and the milt was collected from the sperm duct in order to avoid the contamination or early activation by urine [32].

2.3. Semen quality evaluation - CASA analysis

The CASA analysis was conducted at Department of Animal Physiology using the Computer Assisted Sperm Analyzer method with SpermVision software (Minitub, Tiefenbach, Germany) and the microscope Olympus BX 51 (Olympus, Japan). Semen samples were placed into Makler counting chamber (10 µm, Sefi-Medical Instruments, Germany) [23]. Every single output of the CASA system is the result of 4 diverse sub-measurements of 4 different fields of Makler Counting Chamber. CASA assessments determined the values of total motility (MOT), progressive motility (PRO), distance average path (DAP), distance curved line (DCL), distance straight line (DSL), velocity average path (VAP), velocity curved line (VCL), velocity straight line (VSL), straightness (STR), linearity (LIN), amplitude of lateral head displacement (ALH), beat cross frequency (BCF) of spermatozoa [33–35]. Immotile cells (IMC) were calculated according the following formula: IMC = 100% − MOT.

Table 1
Concentration of selected trace metals in semen samples.

Parameter (unit)	Mean	Median	S.D.	CV%	SEM	Min-max
As (mg/kg)	0.76	0.71	0.49	65.52	0.13	0–1.77
Cd (µg/kg)	22.96	10.14	27.49	119.70	6.87	0–87.73
Cr (mg/kg)	0.18	0.17	0.05	27.34	0.01	0.12–0.32
Cu (mg/kg)	2.16	2.18	0.39	18.52	0.09	1.51–2.89
Fe (mg/kg)	15.51	14.85	5.43	34.99	1.36	8.71–27.92
Hg (µg/kg)	3.54	3.42	1.72	48.65	0.43	1.61–7.18
Mn (mg/kg)	0.44	0.39	0.19	44.89	0.05	0.18–0.82
Ni (mg/kg)	0.48	0.33	0.39	79.97	0.09	0.11–1.36
Pb (mg/kg)	0.25	0.27	0.17	66.81	0.04	0–0.55
Se (mg/kg)	0.33	0.00	0.56	166.15	0.14	0–1.67
Sr (mg/kg)	0.60	0.63	0.38	63.72	0.10	0.19–1.60
Zn (mg/kg)	50.08	51.28	46.03	91.92	11.51	6.59–177.57

S. D. - standard deviation, CV% - coefficient of variation, SEM - standard error, Min -minimum and max – maximum.

2.4. Measurements of RedOx status in semen (ROS, TAC, PC and MDA)

ROS production in samples was quantified by the chemiluminescence assay based on the luminol (5-amino-2, 3-dihydro-1, 4-phthalazinedione; Sigma-Aldrich) probe [36]. Luminol (2.5 µL, 5 mmol/L) was added to 100 µL of semen prior to sample analysis. Negative controls were prepared by replacing the semen with 100 µL of PBS (Dulbecco's Phosphate Buffer Saline without calcium chloride and magnesium chloride; Sigma-Aldrich). Positive controls consisted of 100 µL PBS, 2.5 µL luminol and 50 µL hydrogen peroxide (H_2O_2, 30%; 8.8 M; Sigma-Aldrich). Chemiluminescence was measured in 96-well plates in 15 replicates of 1 min long cycles of Glomax Multi + Combined Spectro-Fluoro Luminometer (Promega Corporation, Madison, WI, USA) set up [37,38]. The results are expressed as relative light units (RLU)/s/g protein.

Total antioxidant capacity (TAC) was determined applying the improved chemiluminescence antioxidant assay which utilizes the horseradish peroxidase conjugate and luminol [39]. 5–100 µmol/L Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; Sigma-Aldrich) was used as the standard and a signal reagent, consisting of 0.1 mol/L Tris-HCl (Sigma-Aldrich), 12 mol/L H_2O_2 (Sigma-Aldrich), 41.8 mmol/L 4-iodophenol (Sigma-Aldrich) and 282.2 mmol/L luminol (Sigma-Aldrich), was employed to induce the chemiluminiscent reaction. Chemiluminescence was quantified on 96-well plates in ten consecutive one minute long cycles using the Glomax Multi + Combined Spectro-Fluoro Luminometer (Promega Corporation). The results are expressed as µmol Trolox Eq./g protein.

Carbonyl group quantification was carried out through the conventional 2,4-dinitrophenylhydrazine (DNPH) method. Briefly, 1 mL of sample was added to 1 mL of DNPH (10 mM in 2 NHCl; Sigma-Aldrich), mixed, and incubated in the dark at room temperature for an hour. Following the addition of 1 mL of trichloroacetic acid (20% w/v; Sigma-Aldrich), the mixture underwent the 10 min of incubation at 4 °C and

Table 2
Sperm quality, DNA fragmentation and RedOx status of semen samples.

	Mean	Median	S.D.	CV%	SEM	Min-max
Sperm quality parameters						
Concentration (10^9/mL)	3.10	3.26	1.39	44.96	0.35	0.99–5.56
Total motility (%)	91.45	91.58	4.89	5.34	1.22	80.20–97.54
Progressive motility (%)	39.47	36.09	26.12	66.16	6.53	2.43–78.75
Immotile cells (%)	8.55	8.42	4.89	57.13	1.22	2.46–19.80
DAP (μm)	15,40	15,50	4,50	29,19	1,12	8,13–26,93
DCL (μm)	21.57	21.47	4.08	18.89	1.02	9.32–28.54
DSL (μm)	13.18	13.00	4.59	34.80	1.15	7.34–25.93
VAP (μm/s)	35,38	35,24	10,99	31,06	2,75	20,38–64,62
VCL (μm/s)	49.05	48.85	9.61	19.59	2.41	23.15–68.47
VSL (μm/s)	30.38	30.16	11.16	36.73	2.79	15.80–62.34
STR (VSL/VAP)	0.84	0,83	0,06	6,65	0,01	0,76–0,96
LIN (VSL/VCL)	0.61	0.62	0.16	25.79	0.04	0.36–0.90
ALH (μm)	2.12	2.26	0.59	28.16	0.15	1.23–2.95
BCF (Hz)	24,84	25,74	4,96	16,96	1,24	9,26–30,80
DNA fragmentation (%)	5.02	5.26	0.66	13.12	0.17	4.00–6.29
Spermatozoa RedOx status						
ROS (RLU/s/g TP)	13.23	9.51	10.27	77.67	2.57	4.54–35.60
TAC (eq. μmol Trolox/g TP)	30.18	30.70	4.47	14.82	1.12	21.52–37.20
MDA (μmol/g TP)	2.32	1.94	1.25	54.04	0.32	0.94–4.72
PC (nmol/mg TP)	1.99	2.24	0.52	26.22	0.130	0.76–2.44

DAP – distance average path, DCL – distance curved line, DSL – distance straight line, VAP – velocity average path, VCL – velocity curved line, VSL – velocity straight line, STR – straightness, LIN – linearity, ALH – amplitude of lateral head displacement, BCF – beat cross frequency.

Table 3
Statistically significant correlations among the trace elements concentrations in semen samples.

Trace elements	*r* (*p* value)
Cu-Fe	0.669 (0.005)
Fe-Mn	0.743 (0.001)
Fe-Sr	0.533 (0.034)
Fe-Zn	0.506 (0.045)
Mn-Se	0.562 (0.023)
Mn-Sr	0.753 (0.0008)
Mn-Zn	0.741 (0.0005)
Se-Sr	0.677 (0.002)
Se-Zn	0.688 (0.003)
Sr-Zn	0.959 (0.0000)

r - Pearson's correlation coefficient.

was centrifugated at 11,828 × *g* for 15 min afterwards. The supernatant was discarded and the undisturbed pellet was three times washed with 1 mL of ethanol/ethyl acetate (1/1; v/v) to remove free DNPH reagent. Further, the pellet was resuspended in 1 mL of 6 M guanidine-HCl (Sigma-Aldrich) and measured by Multiskan FC microplate photometer (Thermo Fisher Scientific Inc.) at absorbance of 360 nm. The molar absorption coefficient of 22,000 1/M.cm was used to calculate the concentration of protein carbonyls groups. Protein carbonyls (PC) are expressed as nmol/g protein [40].

Lipid peroxidation (LPO) was determined by the quantification of malondialdehyde (MDA) production using the TBARS assay, modified for a 96-well plate and ELISA reader. Samples were treated with 5% sodium dodecyl sulfate (SDS; Sigma-Aldrich), and exposed to 0.53% thiobarbituric acid (TBA; Sigma-Aldrich) dissolved in 20% acetic acid adjusted with NaOH (Centralchem) to pH 3.5, and afterwards boiled at 90–100 °C for an hour. Consequently, the reaction was stopped after placing the samples on ice for 10 min. The samples were centrifuged at 1750 × *g* for 10 min and supernatant was used for measurement of the end-product – MDA. Using the Multiskan FC microplate photometer (Thermo Fisher Scientific Inc.), the samples were measured at 530–540 nm [37,38,41]. Concentration of MDA is expressed as μmol/g protein.

Protein concentration was quantified on the semi-automated photometric analyzer Randox RX Monza (Crumlin, United Kingdom) using the DiaSys Total Protein commercial kit according to manufacturers (DiaSys, Holzheim, Germany) recommendation at 540 nm [31,42].

2.5. Evaluation of DNA fragmentation in spermatozoa

DNA fragmentation was evaluated using the APO-DIRECT™ kit (BD Biosciences, Franklin Lakes, NJ, USA). One million cells were collected from each sample, fixed in 4% paraformaldehyde (Centralchem) and incubated on ice for 1 h. Subsequently the cells were washed 3 times in PBS (Sigma-Aldrich) and stored in 70% ice-cold ethanol (Centralchem) overnight at −20 °C. Following storage, the cells were washed, labeled using the DNA labeling solution, rinsed and centrifuged at 300 × *g* for 5 min twice. The pellet was subsequently incubated in PI/RNase staining buffer for 30 min in the dark. Following incubation, each sample was counterstained with DAPI (Sigma-Aldrich) and placed into a black 96-well plate. Appropriate fluorescent signals were obtained using the Glomax Multi + Combined Spectro-Fluoro-Luminometer (Promega).

2.6. Detection of trace elements

Trace metals (As, Cd, Cr, Cu, Fe, Mn, Ni, Pb, Se, Sr and Zn) content was determined in fish milt samples by inductively-coupled plasma optical emission spectrometry (ICP-OES) while Hg content was defined by cold-vapor atomic absorption spectroscopy (CV-AAS).

2.6.1. Pre-analytical procedure for ICP-OES

All the chemicals used during the sample preparation were highly pure. Until the processment of the samples biological material was stored in freezer at −20 °C. The thawed samples (up to 0.5 g) were mineralized in the high performance microwave digestion system Ethos UP (Milestone Srl, Sorisole, BG, Italy) in a solution of 5 mL HNO_3 ≥69.0% (TraceSELECT®, Honeywell Fluka, Morris Plains, USA) and 1 mL of H_2O_2 ≥30%, for trace elements analysis (Merck Suprapur®). Samples, including the blank sample, were digested according to method for animal tissue developed and recommended by

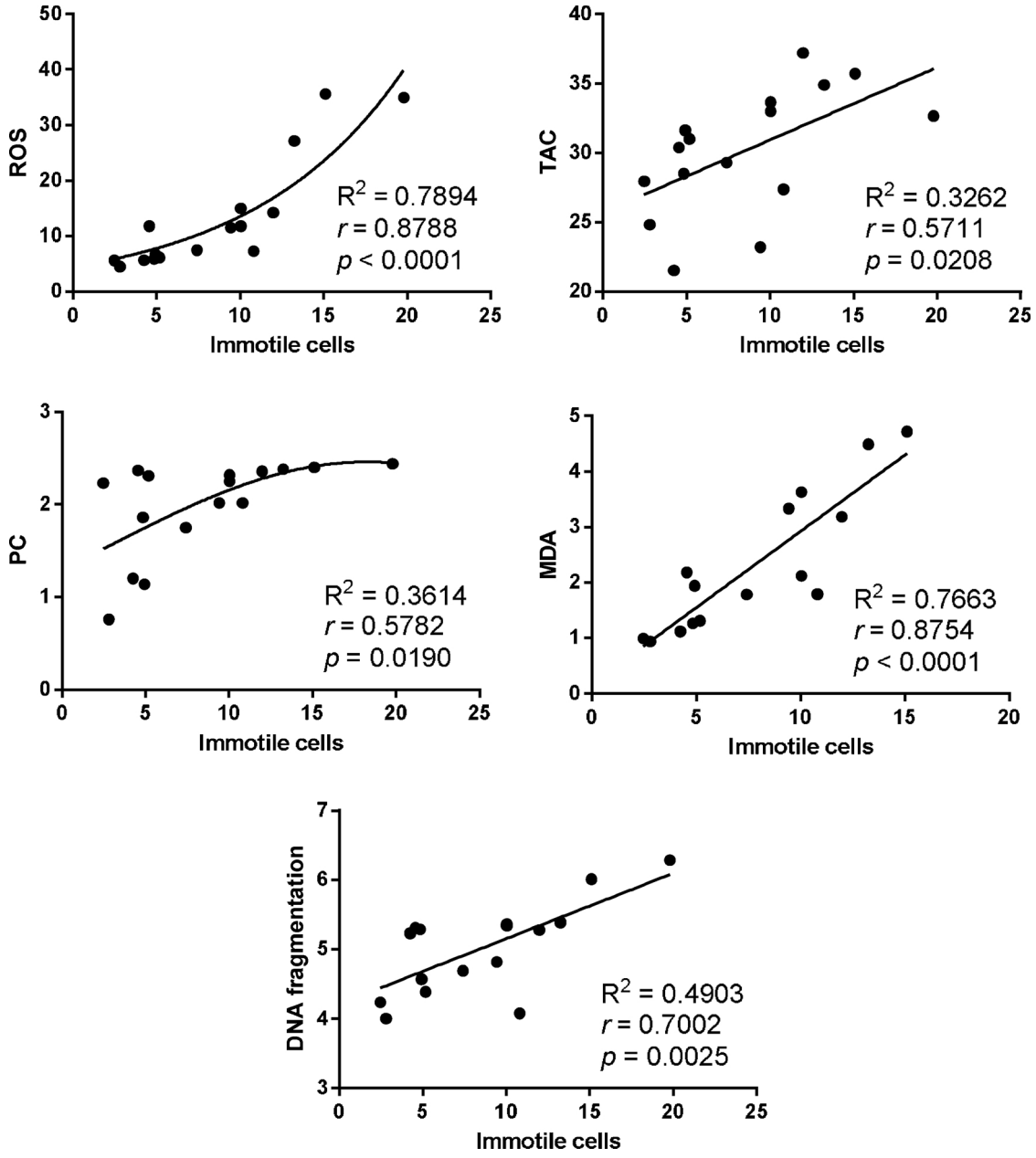

Fig. 1. Dependency between the number of immotile cells and RedOx status parameters and DNA fragmentation. Pearson correlation (*r* - Pearson's correlation coefficient) was carried out to assess this relationship. (The percentage of motile cells was measured in fresh semen; immotile cells (IMC) are a calculated parameter; RedOx parameters concentrations were measured in sperm lysates; each sample was analyzed at least in triplicate). The straight/curve line represents the best fit line obtained by linear or non-linear regression.

manufacturer to achieve the most reliable results. The method consists of heating and cooling phases. During the heating stage the samples were 15 min warmed to 200 °C and this temperature was maintained for another 15 min. Afterwards, during the cooling phase, the samples underwent the 15 min of active cooling to reach the temperature of 50 °C. The digests were filtered through the Sartorius filter discs (grade 390) (Sartorius AG, Goettingen, Germany) into the volumetric flasks and filled up with ultrapure water to a volume of 50 mL [31].

2.6.2. ICP-OES analysis

Quantification of the elements (As, Cd, Cr, Cu, Fe, Mn, Ni, Pb, Se, Sr and Zn) present in milt was conducted using inductively coupled plasma - optical emission spectrophotometer (ICP OES 720, Agilent Technologies Australia (M) Pty Ltd.). Detections limits (μg/L) of measured trace elements were follows: As 1.50, Cd 0.05, Cr 0.15, Cu 0.30, Fe 0.10, Mn 0.03, Ni 0.30, Pb 0.80, Se 2.00, Sr 0.01, Zn 0.20. ICP-OES measurement parameters were follows: RF power 1.30 kW; Plasma flow 15.0 L/min; Auxiliary flow 1.50 L/min; Nebulizer flow 0.85 L/min; Replicated read time 5.00 s; Instrument stabilization 15 s; Sample uptake delay 25 s; Pump rate 15 rpm; Rinse time 10 s; Element wavelengths (nm): As 188.980, Cd 226.502, Cr 267.716, Cu 324.754, Fe 234.350, Mn 257.610, Ni 231.604, Pb 220.353, Se 196.026, Sr 407.771, Zn 206.200 [31]. Multielement standard solution V for ICP (Sigma-Aldrich Production GmbH, Switzerland) was used in this experiment. The concentration of Fe in multielement stock solution was 100 mg/L and other elements (Cd, Cr, Cu, Mn, Ni, Pb, Sr, Zn) 10 mg/L. Concentration of mono-element standard stock solutions for As and Se was 1000 mg/L. The calibration (working) solution for all elements was 0.01, 0.10, and 1.00 mg/L respectively. The legitimacy of the whole method was verified using the certified reference material (CRM – ERM

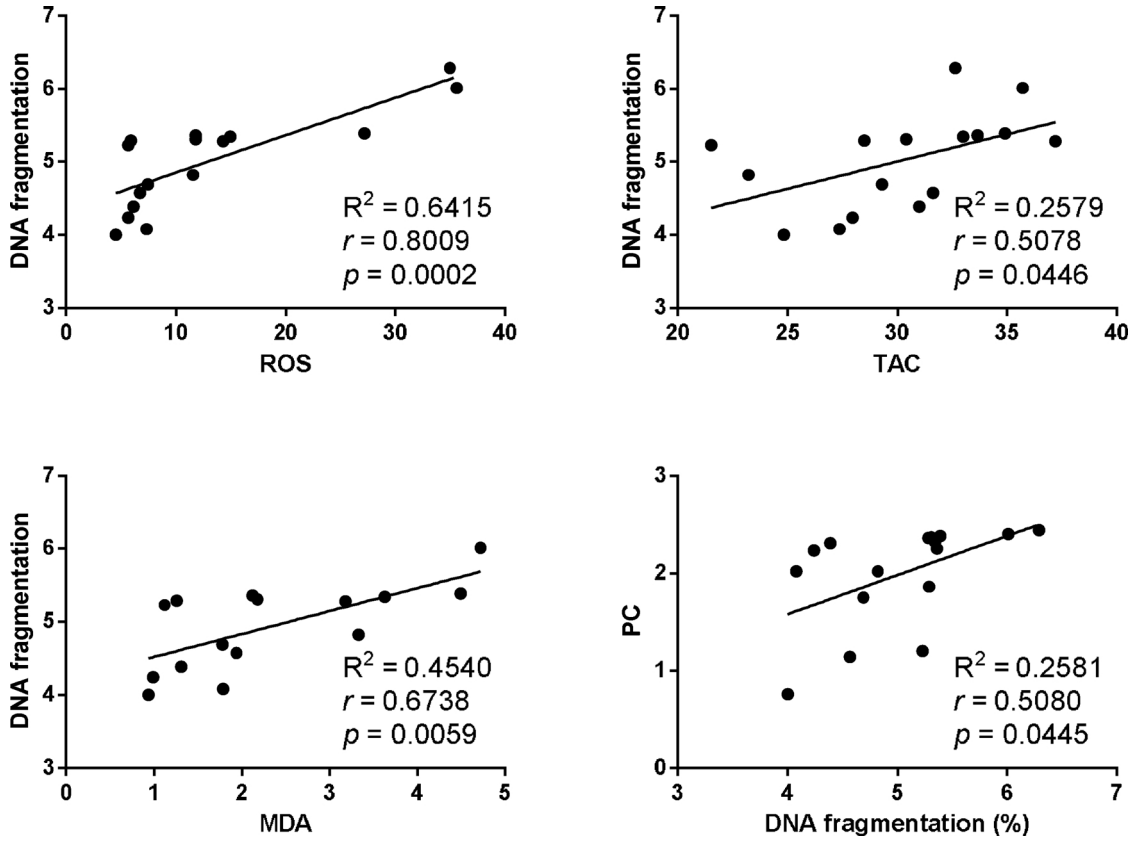

Fig. 2. Dependency between DNA fragmentation and RedOx status parameters. Pearson correlation (*r* - Pearson's correlation coefficient) was carried out to assess this relationship. The straight line represents the best fit line obtained by linear regression.

CE278 K, Sigma-Aldrich Production GmbH, Switzerland).

2.6.3. CV-AAS analysis

Total mercury content (Hg) was analyzed in the thawed semen samples. Selective mercury analyzer AMA-254 (Altec, Praque, Czech Republic) based on CV-AAS was employed with detection limit 1.5 ng/L [31,42].

2.7. Statistical analyses

Statistical analyses were performed using the software systems STATGRAPHICS Centurion (© StatPoint Technologies, Inc., USA) and GraphPad Prism 6.01 (GraphPad Software Incorporated, San Diego, California, USA). Descriptive analysis included calculation of mean, median, standard deviation, coefficient of variation, standard error, and minimum-maximum values. All obtained data were tested for normality using Kolmogorov-Smirnov and Shapiro-Wilk test. The relationship among metals and RedOx status markers and sperm quality parameters were evaluated using Pearson Correlation. $P < 0.05$ was considered to be significant. The regression equations and coefficient of determination were used to evaluate the dependence of sperm quality parameters on RedOx status markers. Correlations of the selected parameters were evaluated at five levels [43]: very weak (0.00–0.19), weak (0.20–0.39), moderate (0.40–0.59), strong (0.60–0.79), and very strong (0.80–1.00). Heatmap with clustering [44] was performed to visualize interactions (Pearson correlations coefficients - *r*) between trace metals concentrations and sperm quality/RedOx status markers. As a clustering method was used an average linkage while the distance measurement method was Euclidean.

3. Results

3.1. Concentration of trace metals

In this study we assessed the relationship between trace elements content and their interactions with the sperm quality and RedOx status in common carp. Mean concentrations of selected trace metals are presented in Table 1 (mean values, median, standard deviations, coefficient of variation, standard errors, minimum and maximum). The most accumulated metal in semen samples was Zn (6.59–177.57 mg/kg). The general scheme of descending concentrations of trace metals in semen samples was following: Zn > Fe > Cu > As > Sr > Ni > Mn > Se > Pb > Cr > Cd > Hg.

3.2. Sperm quality and semen RedOx status

Assessment of sperm quality was realized using CASA system. Mean values of observed parameters are presented in Table 2 (mean values, median, standard deviations, coefficient of variation, standard errors, minimum and maximum). Total motility of spermatozoa was relatively high (91.45%), however progressive motility was not even half of this value (39.47%). Sperm DNA fragmentation was also measured in the same samples of semen and measured values were relatively low (4.00–6.29%). Oxidative status parameters (ROS, TAC, MDA, and CP) are also presented in Table 2.

3.3. Correlation and regression analyzes

Correlation analysis showed some relations between analyzed trace elements in semen samples (Table 3). Very strong (0.959) statistically significant (0.0000) correlation between Sr and Zn was detected. Strong positive statistically significant correlations were observed between Cu

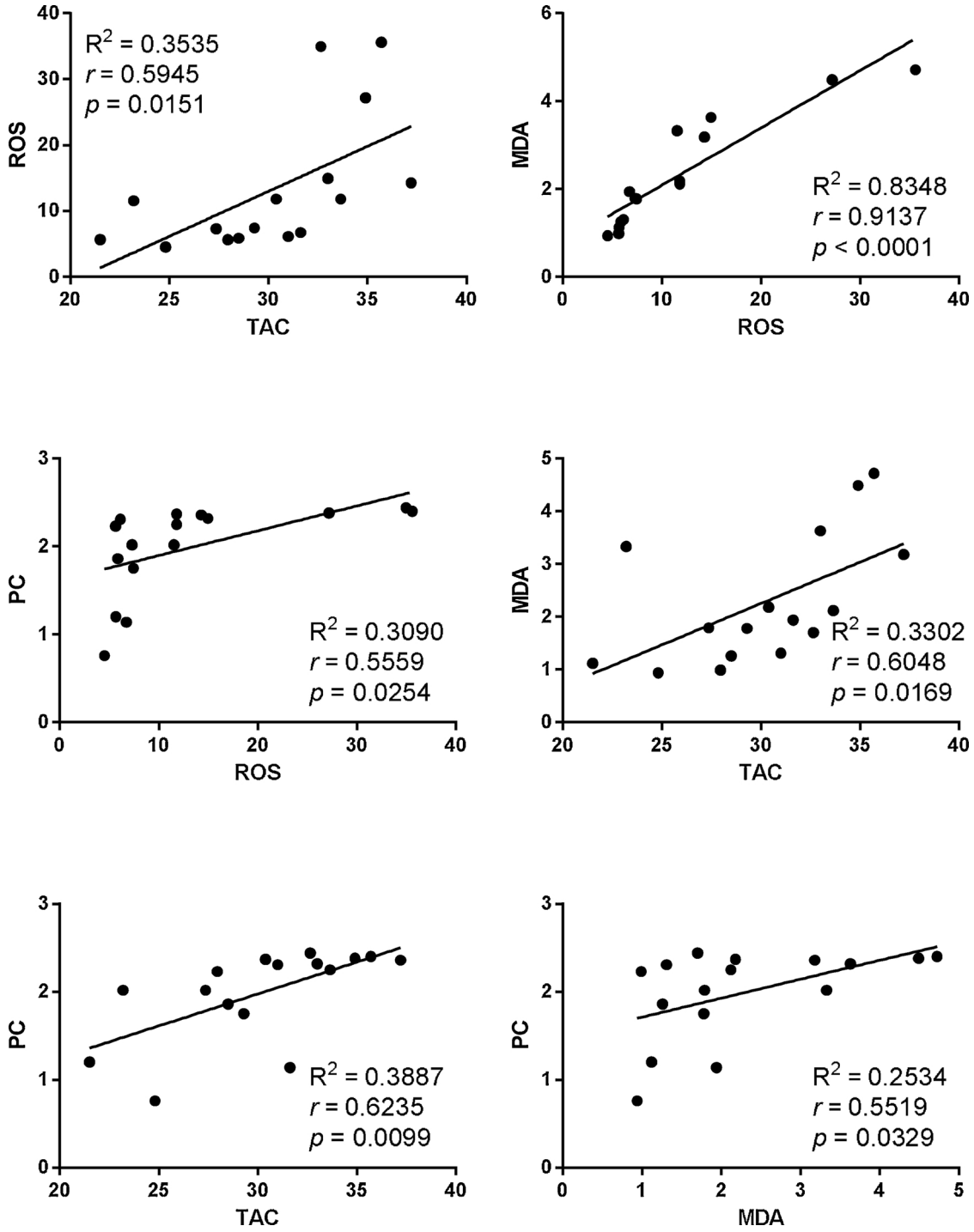

Fig. 3. Relationship between RedOx status parameters. Pearson correlation (*r* - Pearson's correlation coefficient) was carried out to assess this relationship. The straight line represents the best fit line obtained by linear regression.

and Fe (0.669), Fe and Mn (0.743), Mn and Sr (0.753), Mn and Zn (0.741), Se and Sr (0.677) and between Se and Zn (0.688). Moderate positive statistically significant correlations were found between Fe and Sr (0.533), Fe and Zn (0.506) and between Mn and Se (0.562).

Dependencies between immotile cells number and RedOx status parameters and DNA fragmentation are listed in Fig. 1. A significant positive correlation between ROS concentration and immotile cells ($p < 0.0001$) was observed. In addition, the TAC, CP and MDA concentrations in semen samples were significantly increased along with the enhanced percentage of immotile cells. DNA fragmentation percentage showed a significantly positive correlation with immotile cells ($p < 0.01$). The degree of impact of oxidative status markers on immotile cells varied as indicated by R^2 value of fitted linear regression equations.

Dependencies between DNA fragmentation and RedOx status are showed in Fig. 2. Strong positive (0.832) statistically significant (0.0002) correlations were observed between ROS and DNA fragmentation. Significant strong positive correlations were detected between DNA fragmentation and MDA (0.743) and TAC (0.653).

Relationship between RedOx status parameters is showed in Fig. 3. Very strong positive statistically significant correlations were observed between ROS and MDA (0.961), ROS and CP (0.824). Strong positive

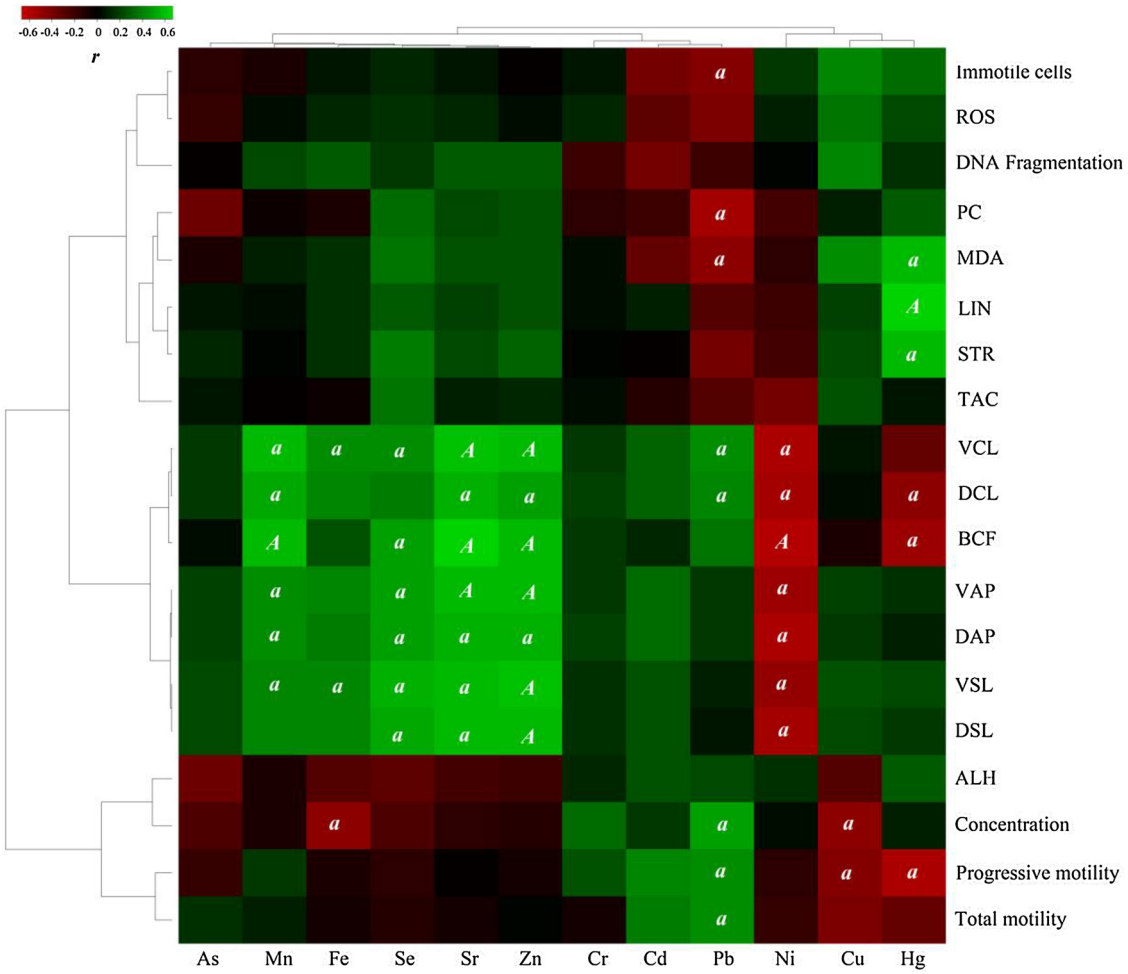

Fig. 4. Heat-map with clustering results (*r* - Pearson's correlation coefficient). Effect of trace metals content on all parameters measured in semen samples of *Cyprinus carpio* (*a* - significant at $p < 0.05$; *A* - significant at $p < 0.01$).

statistically significant correlations were observed between ROS and TAC (0.774), TAC and MDA (0.668), TAC and CP (0.703), and MDA and CP (0.738).

3.4. Heat-map with clustering analysis between trace elements and sperm quality/RedOx status parameters

Heat-map with clustering was used to visualize interactions between trace elements and sperm quality/RedOx status parameters (Fig. 4). This method shows interactions between selected parameters in the clusters, which could explain action of groups of trace metals. Mn, Fe, Se, Sr and Zn show the same tendency with observed parameters (positive association to VAP, VCL, VSL, DAP, DCL, DSL, and BCF; negative association to spermatozoa concentration). The opposite tendency was observed in the Ni concentration. Cu and Hg show similar negative correlation with total motility, progressive motility and BCF; and positive associations with ROS, immotile cells, DNA fragmentation, PC, MDA, STR, and LIN. The opposite tendency was observed in the Pb and Cd concentration.

4. Discussion

Environmental concentrations of heavy metals have been determined as an eventual risk factor to fish overall health status. Synergism of several trace elements and their bioaccumulation may lead to induction of oxidative stress [31], resulting in histological damage [45] and endocrine disruption [46]. Sakkas and Alvarez [47] thoroughly described the origin, distribution and effect of heavy metals on male reproduction. Reproductive system is due to its high sensitivity considered the plausible marker of environment quality [1] what is applicable for mankind [13] as well as for various domestic [3] and wild animals [16]. Fish are especially endangered by the environmental pollution since they are continuously exposed to it. Male gametes production is often directly affected through the alterations in testes [20,29]. Jankovská et al. [48] claim that fish gender is an important environmental factor in respect to the mercury monitoring. Based on their study, the bioaccumulation of Hg in fish gonads is 2.4 times higher in males.

Heavy metals in samples subjected to our experiment were detected and aligned according descending concentrations as follows: Zn > Fe > Cu > As > Sr > Ni > Mn > Se > Pb > Cr > Cd > Hg.

Assessment of the sperm concentration and motility is the essential quality determining factor. Kime et al. [17] describe the CASA system as a reliable tool for fish semen quality evaluation. Dietrich et al. [19] investigated the effect of Hg and Cd (in form of Hg_2Cl_2 and $CdCl_2$) on rainbow trout milt under *in vitro* conditions showing decreasing trend of sperm motility characteristics at concentrations 1 and 10 mg Hg^{2+}/l and 10 mg Cd^{2+}/l. In addition, authors describe lower hatching rates in samples exposed to 10 mg of Hg and Cd ions per liter. Results of present study suggest that Hg negatively affects motility features what is in accordance with the *in vitro* findings however environmental concentration of Cd caused no harm to sperm motility. In addition to Hg, also Cu significantly impaired progressive motility in carp semen. Li et al. [24] exposed sturgeon spermatozoa to environmentally related

and higher concentrations of Cd, Cr and their combination. Neither Cd (0.1 mg/l), Cr (0.001 mg/l) nor combination of these elements (0.1 mg/l Cd + 0.001 mg/l Cr) showed alterations in sperm motility. Although no changes in antioxidant indices were recorded in environmental concentrations of Cd or Cr, their mutual action resulted in enhanced LPO and SOD. According Cabrita et al. [49], oxidative stress markers are very important tool for sperm analysis, especially in terms of toxicity assessment. Intoxication by environmental pollutants stimulates ROS generation and thereby affects motility and DNA integrity [4,7]. Detected significant interactions between individual heavy metals and OS markers emerge as the mechanism responsible for further damage to fish spermatozoa. In our study, positive statistically significant correlations were observed between trace elements (Mn, Se, Sr, and Zn) and qualitative spermatozoa parameters (VAP, DAP, VSL, and BCF). Importance of Zn and Se in seminal plasma was also confirmed by Türk et al. [25] who monitored strong associations with antioxidant activity. Earlier study of Sakkas and Alvarez [47] draws attention to appearance of DNA fragmentation in semen with high concentrations of trace metals. Dietrich et al. [19] associates DNA fragmentation of fish spermatozoa with enhanced values of ROS. We monitored DNA fragmentation on level of 5.02%. These results relate to the study of Nagy et al. [50] who detected DNA fragmentation in 6.09% events in their control group of Prussian carp. Fragmentation of sperm deoxyribonucleic acid, monitored using commercial kit for flow cytometry, reached up to 12.45% fragmentation in experimental samples exposed to various concentrations of Cd (up to 10,000 mg/L). Bergamo et al. [52], defining the semen as biomarker for highly polluted habitat, logically proposed associations between metals content in semen and oxidative stress production, with impact on spermatozoa motility parameters and DNA integrity. The present study confirms this phenomenon with respect to heavy metals induced OS which eventually alters the spermatozoa quality. Even low concentrations of individual trace metals found in the aquatic environment may induce adverse effect in fish reproduction due to strong synergism of several toxicants [24,30].

5. Conclusion

In summary, obtained results imply that chronic exposure to heavy metals polluted environment induces oxidative stress spermatozoa of *C. carpio*. Significant interactions between trace metals (especially Pb and Hg) and MDA (lipid peroxidation) production result in damage of biomolecules. Subsequently, direct interaction was confirmed between RedOx status parameters (especially reactive oxygen species and lipid peroxidation) and DNA fragmentation related to the number of immotile cells. Trace elements, such as Mn, Fe, Se, Sr and Zn showed positive association with spermatozoa qualitative parameters (VCL, VSL, DAP, DCL, DSL, and BCF). Typical toxic element, Hg, negatively affected progressive motility and interacted with production of malondialdehyde, what we can consider as direct effect to lipid peroxidation.

On the basis of previous research, the present study suggests application of multi-component mixtures of environmentally related trace elements concentrations when assessing the potential reproductive risk.

Conflict of interest

The authors declare that there are no conflicts of interest

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