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COMPUTER-ASSISTED ANALYSIS (CASA) OF COMMON CARP *CYPRINUS CARPIO* L. SPERMATOZOA MOTILITY IN THE PRESENCE OF CADMIUM

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ABSTRACT. The effect of different cadmium concentrations on sperm motility in common carp was investigated. The motile activity of spermatozoa was evaluated by means of computer assisted sperm analysis (CASA) using three major parameters characterizing sperm movement – VCL, VAP and VSL. Moreover, subjective microscopic observations were performed in order to evaluate the average time of sperm movement. The following cadmium concentrations were tested: 10, 50 100, 200, 500, 1,000 and 2,000 ppm. Computer assisted analysis and microscopic observations both showed that cadmium decreases the motility of carp spermatozoa in all tested concentrations, and that lethal effects were detectable at a concentration of 500 ppm (as determined by CASA) or 1,000 ppm (when manual microscopic observations were performed). Additionally, it was shown that low cadmium concentrations have the most negative influence on straight line velocity, which suggests the possible negative influence of cadmium on the ability of sperm to fertilize female gametes.

Key words: *CYPRINUS CARPIO*, SPERM MOTILITY, CADMIUM, TOXICITY, CASA

INTRODUCTION

The rapid development of civilization in the last decades of the twentieth century spurred a significant rise in many aspects of economy. Increasing industrialization as well as intensive agriculture has led to the emission of numerous substances that under natural conditions existed only in very limited concentrations. Unfortunately, many of these compounds, or pollutants, have a destructive influence on the environment. This influence is especially visible in the aquatic environment which is the final target of contaminants. They are released in the form of pulp mill to the waters and even if they are emitted to the atmosphere, they finally reach surface waters with acid rains. It can be presumed that the negative influence of pollutants not only contaminate the aquatic environment, but they also influence aquatic organisms such as fish.

Heavy metals are considered as the most dangerous pollutants; among them cadmium is of principal danger. They have a broad spectrum of toxic influence on fish (for review see Jezierska and Witeska 2001). Under natural conditions in Poland,

waters contain very low concentrations of this metal which do not exceed 0.1 μg dm^{-3} (Kabata-Pendias and Pendias 1979). In waters contaminated by the chemical and electrochemical industries, the concentration of this compound can even reach 70 μg dm^{-3} (Szulkowska-Wojaczek et al. 1992).

Although the exact biochemical role of cadmium is not entirely understood, the toxic effects of this metal are well described. Among the various physiological effects, cadmium disturbs many reproductive mechanisms in fish (for a review see Kime 1998). In males it contributes to the degeneration of the testes and influences the seasonal cycle of androgen levels in the blood plasma of brook trout *Salvelinus fontinalis* (Mitchill) (Sangaland and O'Halloran 1973, Sangalang and Freeman 1974). In rainbow trout *Oncorhynchus mykiss* (Walbaum), cadmium concentrations of 0.05 mM disturbed androgen production, but did not have any significant effects at a concentration which was 10 times higher (Kime 1984). It was suggested that cadmium had a stimulatory influence on the synthesis of androgens, but, on the other hand, this metal had a destructive impact on certain tissues and enzymes.

At least in part, cadmium can influence the reproductive systems of fish by competing with calcium in the absorption in gills, since cadmium is absorbed through calcium channels (Verbost et al. 1989).

The main goal of the development of the reproductive system in animals is the production of fertile gametes. Thus, one of the most important factors responsible for the success of reproduction is the quality of male gametes. This implies not only the quality of the genetic material which is introduced into the egg during fertilization, but also the ability of sperm to move toward the female gametes, i.e. sperm motility. Earlier analysis of fish spermatozoa motility used manual microscopic observations. However, in the last decade computer assisted sperm analysis (CASA) has been introduced which permits more information on the different parameters of fish sperm motility to be acquired (Toth et al. 1995, Ebrahimi et al. 1996, Kime et al. 1996). This technique can be used not only to analyze the parameters of movement of intact sperm, but also to determine the changes in sperm motile activity which are caused by different pollutants.

The aim of this study was to evaluate the impact of different concentrations of cadmium on the different parameters of common carp *Cyprinus carpio* L. sperm motility using CASA and to compare this method with subjective microscopic observations of spermatozoa motility. Recently, we have demonstrated that sperm motility

evaluation using CASA is very advantageous and provides more precise and detailed information concerning the different parameters of its motility (Chyb et al. 2000, Epler et al. 2000).

MATERIAL AND METHODS

ANIMALS

The experiment was conducted in June in the Department of Ichthyobiology and Fisheries, Agricultural University of Kraków, Poland. Seven mature, spermiating male carp were used in the study. Prior to the study, the fish were caught from earth ponds and transferred to concrete flow-through basins. Then fish were acclimated for at least three days to 20°C under a simulated natural photoperiod.

SPERM SAMPLING

Fish were anaesthetized for 5 minutes using 2-phenoxyethanol (3 ml for 10 dm^{-3} of water), wiped with a wet cloth and stripped of milt using a 1 ml sterile syringe. The sperm collected from each fish was then immediately diluted with different pollutant concentrations.

EXPERIMENT DESIGN

The two-step method of sperm dilution according to Billard and Cosson (1992) was used in the experiment. In the first step, the sperm was diluted 100-fold in 10-ml polypropylene tubes in a basal diluent (control group) consisting of KCl 200 mM - Tris 30 mM pH = 8.0 or in a basal diluent containing the following concentrations of pollutant - $CdCl_2$ (P.O.Ch., Poland) - 10, 50, 100, 200, 500, 1,000 and 2,000 ppm (expressed as the concentration of pure metal). After gentle mixing, the tubes were incubated for 2 h at 4°C. In the second step, just before recording sperm motility, 1 µl of each mixture was placed on a polyvinyl alcohol (PVA) coated microscope slide (Chance Propper Ltd., UK) and then quickly mixed with 20 µl of distilled water for a total dilution of 2,100 times.

COMPUTER ASSISTED SPERM ANALYSIS (CASA)

Sperm movement was recorded on a VHS video cassette recorder (VQ 336, Samsung, South Korea) for two minutes from the moment of final dilution using a videocamera (TK 1280E, JVC, Japan) attached to an inverted microscope with a

phase-contrast objective lens (Wilovert S, Hund, Germany, total magnification – 100x). Videotapes were analysed using a Hobson sperm tracker (Hobson Tracking Systems Ltd., Sheffield, U. K.), from 20 seconds after the mixing point (to allow for focusing and stabilization of water solution movement) for a 15 second period. The following parameters were analyzed: VCL - curvilinear velocity ($\mu m\ s^{-1}$), the sum of the incremental distances moved in each frame along the sampled path divided by the total time of the track; VSL - straight line velocity ($\mu m\ s^{-1}$), the straight line distance between the start and end points of the track divided by the time of the track; VAP - angular path velocity ($\mu m\ s^{-1}$), a derived path based on an average number of points and divided by the time of the track. More details of the analysis are given in Kime et al. (1996).

TIME OF SPERM MOTILITY

The time of sperm motility was subjectively measured using a timer, from the moment of sperm activation until about 80% of the sperm did not exhibit motile activity.

STATISTICS

The data on VCL, VAP, VSL and average time of sperm motility (MOT) was analysed using the analysis of variance followed by Duncan's multiple range test. The differences between control and experimental groups were considered significant for $P < 0.05$.

RESULTS

CURVILINEAR VELOCITY (VCL)

In all concentrations used in the experiment, cadmium significantly decreased the curvilinear velocity of common carp spermatozoa (Fig. 1). In the lowest cadmium dose of 10 ppm, the curvilinear velocity (VCL) value reached $47.00 \pm 2.72\ \mu m\ s^{-1}$ versus $86.84 \pm 2.67\ \mu m\ s^{-1}$ in the control group. The higher doses caused the subsequent dose dependent decrease of VCL values until the 500 ppm dose where no sperm motility was detected.

AVERAGE PATH VELOCITY (VAP)

The average path velocity in the control group was $69.77 \pm 1.92\ \mu m\ s^{-1}$. All concentrations of cadmium used in the study decreased the values of the VAP parameter in a

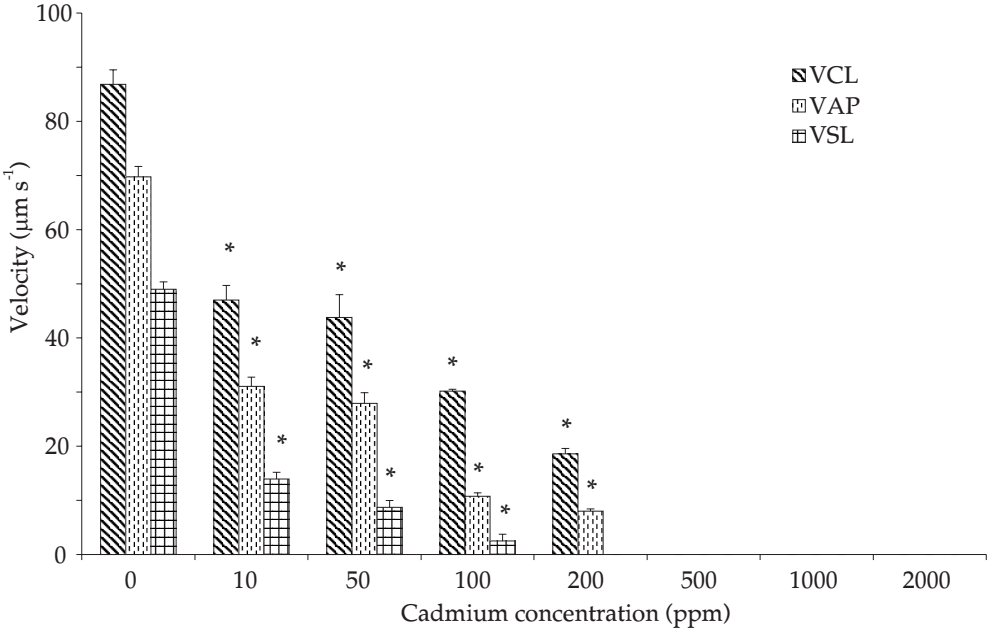

Fig. 1. The effects of different cadmium concentrations on curvilinear velocity (VCL), average path velocity (VAP) and straight line velocity (VSL) of common carp sperm. The values are expressed as mean (main bars) ± SEM (error bars). * - statistically different vs. appropriate control group ($P < 0.05$).

dose-dependent manner (Fig. 1). Cadmium concentrations of 500, 1,000 and 2,000 ppm completely blocked the VAP motility of carp spermatozoa.

STRAIGHT LINE VELOCITY (VSL)

Similarly to the parameters presented above, the cadmium ions significantly decreased the VSL parameter of carp sperm motility in a dose-dependent manner starting from a concentration of 10 ppm where the straight line velocity was 14.84 ± 0.79 μm s^{-1} versus 49.01 ± 1.36 μm s^{-1} in the control group (Fig. 1). Cadmium concentrations of 500, 1,000 and 2,000 ppm completely stopped sperm motility measured by the VSL parameter.

TIME OF SPERM MOTILITY (MOT)

The microscopic observations of sperm motility after incubation in different concentrations of cadmium showed that this metal significantly decreased motility in all concentrations except that of 10 ppm (Fig. 2). Moreover, the lack of motility was only observed in the highest two concentrations of cadmium (1,000 and 2,000 ppm).

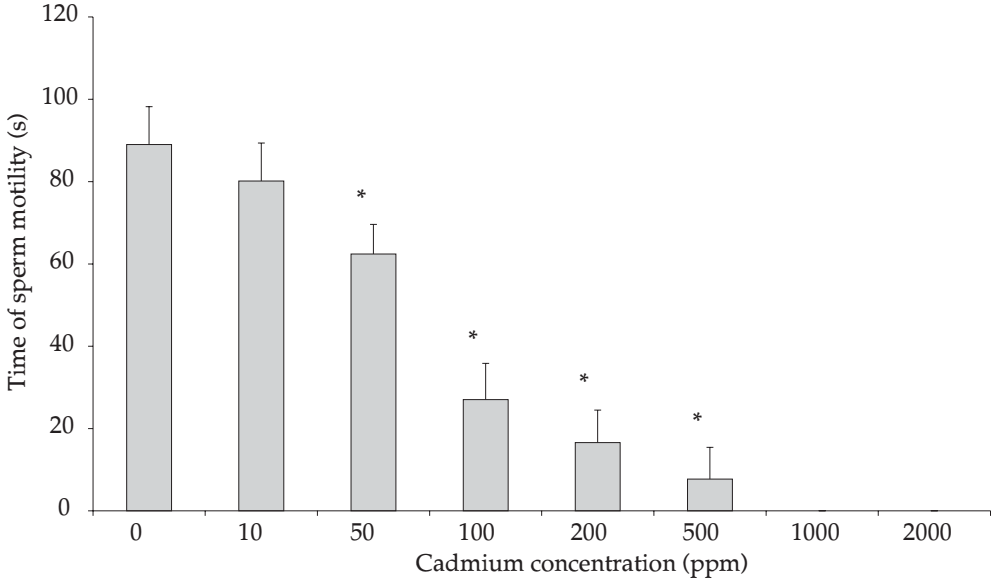

Fig. 2. Effects of different concentrations of cadmium on the average time of carp sperm motility. The values are expressed as mean (main bars) ± SEM (error bars). * - statistically different vs. appropriate control group ($P < 0.05$).

DISCUSSION

Recent years have seen a heightened sense of ecological social consciousness in developed countries. As a consequence, there has been a drop in pollution levels in many countries, including Poland. However, industrial and agricultural pollutants, for example cadmium, which contaminate inland waters are still observed in concentrations that are several-fold higher than naturally occur. Even if these concentrations are not lethal, they can influence many aspects of fish physiology, including reproduction, as they are accumulated in different tissues. Thus, our goal was to determine the impact of sublethal cadmium concentrations on carp sperm motility.

All the computer-analyzed parameters of sperm motility indicated that cadmium significantly decreased the motility of carp sperm even at a concentration of 10 ppm. This negative influence was dose-dependent. The motility of sperm was completely blocked at concentrations of 500, 1,000 and 2,000 ppm. The negative action of cadmium could be the result of the displacement of calcium from its specific binding sites in spermatozoa, since calcium channels are the targets for many heavy metals

(Büsselberg 1995). The displacement of calcium by cadmium ions in spermatozoa was also observed by Ebrahimi et al. (1996) in African catfish *Clarias gariepinus* (Burchell). Since calcium is a necessary element for sperm movement (Maisse et al. 1995), the displacement of calcium ions by cadmium would be the factor responsible for the inhibition of motility. Furthermore, the dynamics of the decrease of the motility in the case of these three parameters is similar. However, there are some differences in the decrease of the velocity in the presence of low concentrations of cadmium. The decrease in straight line velocity at a concentration of 10 ppm (30% of the value observed in the control group) is much higher than that of curvilinear velocity (54% of control value). Thus, cadmium ions would more significantly influence VSL than VCL. The findings of Moore and Akhondi (1996) indicate that the success of fertilization depends on VSL. This, in turn, suggests that sublethal cadmium concentrations can greatly affect the ability of carp spermatozoa to fertilize eggs.

Similar investigations on the effects of cadmium on sperm motility performed on African catfish (Ebrahimi et al. 1996), showed that cadmium negatively influenced the VCL, VAP and VSL in similar concentrations to those used in the present study. This was so despite the different incubation times - catfish sperm was exposed to the pollutant for 24 h, while carp were only exposed for 2 h.

It is difficult to explain this phenomenon, but it is possible that carp has much better ability to accumulate cadmium ions than catfish does, as is the case with zinc. Zinc is accumulated about 15-fold more effectively by carp than by other freshwater species – grass carp *Ctenopharyngodon idella* (Val.) or silver carp *Hypophthalmichthys molitrix* (Val.) (Jeng and Sun 1981).

Slightly different results to those obtained using computer assisted analysis were found when the average time of sperm motility was measured by means of microscopic observations. Although it was found that cadmium decreases the time of sperm motility, this negative influence was not visible in all the tested cadmium concentrations as was the case when computer-assisted analysis was applied. Cadmium at the concentration of 10 ppm did not have a significant influence on the duration of sperm motility. This is slightly different from the results obtained by Jezierska et al. (1995), where the 2-hour incubation with cadmium at the concentration from 0.005 to 0.5 ppm led to a slight but significant decrease of sperm motility. On the other hand, in the present study, sperm motility was observed at a concentration of 500 ppm, which was found to be lethal when computer analysis was performed. These differences between the results of the two methods of sperm motility analysis can be attrib-

uted to the false interpretation of sperm motility during manual microscopic observations. This is supported by the fact that the results of all three computer-analyzed parameters (VCL, VAP and VSL) are coincident with each other. This suggestion is supported by the findings of Centola (1996) who has shown that CASA results are much more precise than the manual microscopic technique of evaluating sperm motility.

In conclusion, cadmium negatively influenced carp sperm motility, decreasing different parameters of sperm motility as well as the overall time of sperm movement. The computer assisted sperm analysis indicated that the toxicity of cadmium was higher than did analysis performed using the traditional method.

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STRESZCZENIE

KOMPUTEROWA ANALIZA RUCHLIWOŚCI PLEMNIKÓW KARPIA *CYPRINUS CARPIO* L. W OBECNOŚCI KADMU

W niniejszej pracy badano wpływ różnych stężeń kadmu na ruchliwość plemników karpia. Aktywność ruchową plemników oceniano za pomocą wspomaganej komputerowo analizy plemników (CASA) z wykorzystaniem trzech parametrów charakteryzujących poruszanie się plemników – VCL, VAP i VSL. W ramach niniejszych badań mierzono także czas ruchliwości plemników metodą obserwacji mikroskopowych. W doświadczeniach zastosowano następujące stężenia kadmu: 10, 50 100, 200, 500, 1000 oraz 2000 ppm. Zarówno komputerowa analiza ruchliwości, jak i obserwacje mikroskopowe wykazały, że kadm hamuje ruchliwość plemników wykazując efekt letalny przy koncentracji 500 ppm (w przypadku metody komputerowej CASA - rys. 1) lub 1000 ppm (w przypadku subiektywnej oceny czasu trwania ruchu plemników - rys. 2). Wykazano ponadto, że niskie stężenia kadmu w największym stopniu obniżają ruchliwość plemników mierzoną parametrem VSL, co sugeruje prawdopodobny negatywny wpływ tego metalu na zdolność plemników do zaplemnienia.

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