



**DEVELOPMENT OF TOXICOLOGICAL TEST SYSTEMS BASED ON  
FISH SPERM ANALYSIS**

PhD thesis

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## 1. Introduction and aims

The number of vertebrates used for scientific purposes is estimated at 100 million every year. According to the 7<sup>th</sup> report of the European Committee, 11.5 million vertebrates were used for animal experiments in 2013. Due to this, the aim of the EU is to reduce the number of animals used for scientific purposes through the 3R strategy (Reduction, Replacement, Refinement) and to replace the experiments carried out on vertebrates with alternative methods which give reliable and accurate results. The reduction of suffering of experimental animals to a minimal level is also an important issue as well as the standardization of experimental conditions, allowing the comparison of results among laboratories, which also contributes to the principle of the reduction.

Due to their ethical and practical advantages, *in vitro* test systems are also widespread in ecotoxicological studies these days. In addition, the Organisation for Economic Co-operation and Development has published numerous guidelines related to toxicological tests, which serve to test the harmful effects of xenobiotics, ensuring that the circumstances of experiments are standard in every detail. Fish are important model organisms in ecotoxicological studies. As their lifestyle is bound to water, they are directly exposed to environmental pollutants. Due to this, in these guidelines zebrafish (*Danio rerio*) and common carp (*Cyprinus carpio*) are listed as suggested model species for the experiments. Fish sperm could be a very good *in vitro* toxicological model, due to its parameters which allow easy, quick and objective measurement, which makes the exposure of living animals unnecessary; despite this, the examination of fish sperm is not mentioned in any of the guidelines. In addition, fish sperm – contrary to cell cultures used generally – does not need maintenance, it can be stripped from the donor whenever, freshly, by a non-invasive method, due to this, its use in toxicological tests is less time-, labour- and cost intensive.

In the last years, many experiments were published in which fish sperm was used as toxicological model, however, the described methods are different in

several aspects. One of the main differences is the fish species supplying sperm, the other is the endpoints investigated in the experiments. Numerous reports are related to the examination of motility parameters of fish sperm, but none of these are uniform: the results of experiments carried out in the same species and with the same toxic solutions are sometimes contradictory because of the different experimental conditions and technical details. Furthermore, several types of damages can affect spermatozoa in case of toxic exposure (e.g. oxidative DNA damage) which do not manifest in the motility parameters of sperm, however, after the fertilization they can cause serious deformities and malformations in the developing embryos, thus, these examinations are relevant.

## 1.1 Objectives

Through my doctoral work, my goal was to develop fast, easy and repeatable *in vitro* toxicological test systems based on fish sperm analysis, which eliminate the technical differences among experiments and allow to compare the results of sperm analysis and measurements carried out in different laboratories. Experiments have been carried out in zebrafish and common carp, the investigated parameters were as follows at different exposure times: progressive motility of sperm (PMOT, %), curvilinear velocity of sperm movement (VCL,  $\mu\text{m/s}$ ), linearity of movement (LIN, %), as well in zebrafish the fertilization rate (%) recorded by exposed sperm, the survival of embryos at 48 hours post fertilization (%) and the rate of embryonic deformities (%). During my experiments, the effect of seven different heavy metals, chromium, zinc, cadmium, nickel, copper, arsenic and mercury on the mentioned parameters was investigated. I was looking for the answers for the following questions:

- Does dose-response and time-response appear on the examined parameters affected by the heavy metal exposure, thus, do the investigated values change proportionately with the increasing of concentration and exposure duration?

- In case of dose-response, is it possible to calculate median effective concentrations (EC<sub>50</sub> values) of the various examined parameters, thus, do the changes allow the toxicants to reach the 50% level in the examined endpoints?
- Applying the same examining protocol in zebrafish and common carp, do the results differ significantly from each other?
- Fertilizing with sperm exposed by heavy metals, does the fertilization rate of zebrafish embryos and the rate of surviving embryos decrease as well? Do deformities and delay in development appear related to the control group?
- Following the exposure of sperm, does motility assesment or does fertilizing capacity prove to be most sensitive?

## 2. Materials and methods

### 2.1 Stripping, dilution and exposure of sperm

In case of zebrafish, I have worked on mixed sperm samples coming from different males. Ten  $\mu\text{L}$  sperm was collected by stripping into 50  $\mu\text{L}$  of cyprinid immobilising solution. In case of common carp, the experiments were carried out on individual, independent samples. Before the experiments – as during the experiments of motility assessment –, the quality of samples was checked by Computer Assisted Sperm Analysis system. The tested concentrations of heavy metals were determined based on previous range finding tests, which were as follows in both species: 50, 100, 150, 200 mg/L in cases of  $\text{Cr}^{3+}$  and  $\text{Zn}^{2+}$ , 1, 5, 25, 50 mg/L in cases of  $\text{Cu}^{2+}$  and  $\text{Cd}^{2+}$ , 600, 800, 1000, 1200 mg/L in case of  $\text{Ni}^{2+}$  and 0.5, 1, 2.5, 5 mg/L in case of  $\text{Hg}^{2+}$ . Only in case of  $\text{As}^{3+}$ , different ranges of concentrations had to be used in the two species: 50, 100, 150 and 200 mg/L were tested on zebrafish sperm, while 1, 5, 25 and 50 mg/L on common carp sperm. In case of common carp, from the freshly stripped sperm, 10  $\mu\text{L}$  was diluted in 50  $\mu\text{L}$  of cyprinid immobilising solution in order to reach the 6-fold dilution rate as in zebrafish. After this, the samples were handled the same way in zebrafish and in common carp, too: 10  $\mu\text{L}$  of diluted sperm was further diluted in 10  $\mu\text{L}$  of immobilising solution containing the toxicant (2-fold dilution) reached the final examining concentrations. In case of every sample, one untreated control group was examined in parallel, which was diluted in cyprinid immobilising solution at the same, 1:1 dilution rate at the beginning of the experiment, in the same time with the toxic dilution of the treated samples. Duration of the exposure to the toxicants was 4 hours in the experiments of sperm motility assessments, while it was 30 and 120 minutes during the investigation of fertilization rate and embryogenesis. During the exposure, the samples were stored on melting ice.

## **2.2 Toxicological-aimed sperm analysis**

The endpoints of sperm analysis were those motility parameters, which were most frequently examined previously in the *in vitro* toxicology studies carried out on fish sperm. These were progressive motility (PMOT, %), one of the velocity parameters (curvilinear velocity, VCL,  $\mu\text{m/s}$ ) and one of the linearity parameters (LIN, %). The recording of these endpoints was conducted at 30-minute intervals of the 4-hour exposure after the dilution with the heavy metal solutions in different concentrations. During sperm analysis, in case of zebrafish, 1  $\mu\text{L}$  of sperm diluted with the toxicant was activated in 2.5  $\mu\text{L}$  system water, while in case of common carp, higher dilution was needed, thus, 0.5  $\mu\text{L}$  of sperm exposed to heavy metals was added to 25  $\mu\text{L}$  system water. In both species, the experiments were carried out on 5 independent samples: in case of zebrafish, 5 different sperm samples coming from different males were used, while in case of common carp, the experiments were carried out on 5 independent sperm samples coming from 5 different males.

## **2.3 Investigation of fertilization and embryogenesis in zebrafish**

In the fertilization experiments, 20  $\mu\text{L}$  immobilised sperm exposed for 30 and 120 minutes to heavy metals in different concentrations was added to the batches of eggs, which were activated by cca. 1 mL of system water. The Petri dishes were incubated at 26 °C for 48 hours, during this period water was replaced once – after 24 hours – on them and non-fertilized eggs as well dead embryos were removed.

The endpoints of the experiments were fertilization rate (%) and the survival of embryos at 48 hours (%) compared to the number of fertilized eggs. The fertilization rate was determined 24 hours after the fertilization. In addition, different deformities and developmental malformations were examined after 24 and 48 hours, during that the development of following deformities were

investigated: sac oedema, pericardial oedema, deformities of head and eye development, vertebral and tail malformations, desintegrated body. The experiments were carried out in 3 replicates in cases of every heavy metal and exposure duration.



### 3. Results

#### 3.1 Toxicological-aimed sperm analysis

In the toxicological-aimed sperm analysis, dose-response was found between the different motility parameters and the applied concentrations of heavy metals in zebrafish as well common carp. However, the level of response was different in the examined endpoints. The progressive motility of sperm (PMOT) proved to be the most sensitive: the triggered effect was the highest here and significant reduction has appeared firstly in this parameter. Regarding the calculated EC<sub>50</sub> values, they can be calculated in most cases for PMOT, however, these sometimes proved to be higher than those EC<sub>50</sub> values which calculated for VCL. Differences can also be observed regarding the EC<sub>50</sub> values calculated for the sperm of two species: in some cases the sperm of zebrafish, while in some other cases of the common carp reacted more sensitively to the exposure to heavy metals. However, in case of As<sup>3+</sup>, the difference was caused by the different concentrations that had to be used due to the difference in sensitivity of the two species against this substance. However, it can be concluded, that while in zebrafish sperm the toxic effect did not manifest immediately after the exposure, then in common carp sperm, a significant reduction could be observed already at the beginning of the experiment, after 30 minutes.

The velocity of sperm (VCL) was less sensitive in cases of both species: the examined concentrations triggered lower response than in case of PMOT. Calculation of EC<sub>50</sub> values was possible in fewer points of exposure duration. In addition, in common carp, calculation of EC<sub>50</sub> values was not possible for each heavy metal treatment (in cases of Cu<sup>2+</sup> and Ni<sup>2+</sup>) at the applied exposure durations. However, it can be said that these values sometimes were lower than those calculated for PMOT. The linearity of sperm (LIN) proved to be the least sensitive parameter in the two examined fish species: dose-response was not observed in cases of many heavy metals (in case of Ni<sup>2+</sup> in neither species, in case

of  $\text{Cd}^{2+}$  in zebrafish). There were situations where an increase of these values was observed (exposure of common carp sperm to  $\text{Cu}^{2+}$ ) and the calculation of  $\text{EC}_{50}$  values was possible only during the investigation of few metals (in case of  $\text{Hg}^{2+}$  in both species, in case of  $\text{As}^{3+}$  only in common carp). During the examination of PMOT, VCL and LIN, a significant interaction was observed between the investigated variables (exposure time and concentration) affected by most heavy metals which can be considered trivial as they have a joint effect, due to this, they strengthen each other.

### **3.2 Investigation of fertilization and embryogenesis in zebrafish**

During the experiments, a significant effect was not observed on the fertilizing capacity of sperm at the applied exposure durations and concentrations in cases of two heavy metals, the  $\text{Cd}^{2+}$  and  $\text{Zn}^{2+}$ . In cases of further 4 heavy metals, during the examination of  $\text{Cr}^{3+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Ni}^{2+}$  and  $\text{Hg}^{2+}$ , the applied concentrations significantly reduced the fertilizing capacity of sperm, but the effect of applied exposure durations was not significant. Only in case of  $\text{As}^{3+}$ , the concentration as well as the exposure time affected the fertilizing ability. The survival of embryos examined at 48 hours and the rate of embryonic deformities did not differ significantly from the control in case of any treatment of heavy metals.

#### **4. New scientific results**

1. A toxicology test system has been developed based on fish sperm analysis with which the effect of seven heavy metals (chromium, zinc, cadmium, nickel, copper, arsenic and mercury) on zebrafish and common carp sperm has been investigated.
2. Evidence was given that zebrafish and common carp sperm react with dose-response and time-response to the toxic exposure to these heavy metals.
3. Evidence was given that among the examined motility parameters, progressive motility of sperm reacts to the exposure to heavy metals the most sensitively in zebrafish and common carp.
4. Median effective concentrations (EC<sub>50</sub> values) on the motility parameters (progressive motility, curvilinear velocity, linearity) of zebrafish and common carp sperm and on the fertilizing capacity of zebrafish sperm followed by exposure to heavy metals have been determined.
5. Evidence was found that upon fertilization with sperm exposed to the examined seven heavy metals the rate of embryonic deformities and the survival does not change significantly until the 48 hours age of embryos.

## 5. Conclusions and suggestions

Comparing my results with those which are presented in the literature, it can be concluded that numerous differences were presented in the results which can be attributed to different conditions of the experiments. However, it seems clear that fish sperm react with dose-response and time-response to the exposure to heavy metals, thus, its measurable parameters are able to indicate its exposure to heavy metals. Due to this, fish sperm can be used as *in vitro* toxicological model which has several advantages over the *in vivo* tests carried out on fish as well the generally used, *in vitro* test systems. Comparison the results of experiments carried out previously presents difficulties because of the different protocols in some cases. In order to bridge this, a uniform method was developed in the present doctoral work which is fast, easy to carry out with considering the animal protection aspects and gives replicable and reliable results. This process can be summarized as follows: (1) dilution of 10  $\mu\text{L}$  (zebrafish or common carp) sperm in 50  $\mu\text{L}$  cyprinid immobilizing solution; (2) further dilution of 10  $\mu\text{L}$  from this diluted sperm at a rate 1:1 with immobilizing solution containing the toxicant in order to reach the final concentration of the tested heavy metal; (3) storage of samples on melting ice for 120-240 minutes of exposure; (4) measurement of the toxicity of tested chemicals by a CASA system or by carrying out fertilizing experiments.

Regarding the exposure duration it can be concluded that experimental variation (expressed in SD) reduced in paralel with time in the most cases, thus, more reliable results are generated at the end of the exposure period which justifies the application of a longer exposure. Also, in some cases, the toxic effect of heavy metals cannot be observed at the beginning of the exposure which also warrants the use of a longer exposure duration.

Comparing the endpoints of experiments followed by toxic exposure of sperm, motility parameters or the fertilizing capacity of sperm reacted sensitively to the toxic exposure in cases of the different heavy metals differentially.

However, in cases of  $\text{Cd}^{2+}$  and  $\text{Zn}^{2+}$ , the fertilizing capacity of exposed sperm was not affected significantly. Furthermore, the assessment of motility parameters has some advantages over the examination of fertilizing capacity: (1) motility assessment can be carried out whenever, while fertilization experiments are possible only when eggs are available; (2) the results of motility assessment do not depend on the quality of eggs, thus, it gives more reliable results; (3) motility parameters are able to indicate the toxic effects to sperm in real-time, while the determination of fertilization rate is possible only 2-3 hours after the fertilization. Despite of this, the fertilization rate is also able to indicate the possible toxic exposure of sperm, thus, its testing can be reasonable in the lack of CASA system.

## 5.1 Suggestions

Based on my results, the following suggestions are made:

- the *in vitro* toxicological-aimed examination of zebrafish and common carp sperm is suggested according to the uniform method developed in the dissertation with which a possible heavy metal pollution of environmental samples can be detected,
- a longer exposure duration (180-240 minutes), yielding more reliable results is suggested,
- the measurement of the progressive motility (PMOT) of sperm as toxicological experimental endpoint is suggested which proved to be the most sensitive endpoint in cases of every examined heavy metal,
- fertilization experiments with exposed sperm according to the developed method are suggested if a CASA system is not available for the detection of sperm motility.

The following suggestions are made regarding future research:

- the examination of the effect of heavy metals on the sperm of fish species belonging to other families is suggested according to the method developed in the dissertation in order to state a sensitivity order among the species,
- investigation of the effect of compounds belonging to other chemical groups is suggested based on the developed protocol in order to allow comparison of the effect of different chemicals on fish sperm.

## 6. Publications related to the topic of the dissertation

### Publications in scientific journals:

**Kollár, T., Kása, E., Ferincz, Á., Urbányi, B., Csenki-Bakos, Z., Horváth, Á.** (2018): Development of an *in vitro* toxicological test system based on zebrafish (*Danio rerio*) sperm analysis. Environmental Science and Pollution Research 25(15): 14426-14436., doi: 10.1007/S11356-018-1613-2.

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**Kollár, T., Gazsi, G., Kása, E., Urbányi, B., Csenki-Bakos, Z., Horváth, Á.** (2018): Investigation of embryogenesis following fertilization with sperm exposed by heavy metals, in zebrafish (*Danio rerio*). Environmental Science and Pollution Research, under review.

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**Kollár, T., Kása, E., Urbányi, B., Csenki-Bakos, Z., Horváth, Á.:** Investigation of embryogenesis followed fertilization with sperm exposed by heavy metals, in zebrafish (*Danio rerio*). VIII International Conference "Water & Fish", Belgrade, Serbia, 13-15 June 2018, book of abstracts p. 282-284.

**Kollár, T., Kása, E., Csenki-Bakos, Z., Horváth, Á.:** Investigation of embryogenesis following fertilization with sperm exposed by heavy metals, in zebrafish (*Danio rerio*). National Conference of Young Biotechnologists, Budapest, Hungary, 28-29 March 2018, book of abstracts p. 71

**Kollár, T.:** Investigation of embryogenesis following fertilization with sperm exposed by heavy metals, in zebrafish. „Excellent Talents of Szent István University” conference, Gödöllő, Hungary, 9 February 2018, book of abstracts p. 66.

**Kollár, T., Kása, E., Urbányi, B., Csenki-Bakos, Z., Horváth, Á.:** Investigation of embryogenesis following fertilization with sperm exposed by heavy metals, in zebrafish (*Danio rerio*). XV. PhD – Conference organized by Professors for European Hungary Association, Budapest, Hungary, 8 November 2017, book of abstracts 2<sup>nd</sup> vol. p. 28-34.

**Kollár, T., Kása, E., Ferincz, Á., Csorbai, B., Urbányi, B., Csenki-Bakos, Z., Horváth, Á.:** *In vitro* toxicology test system based on common carp (*Cyprinus carpio*) sperm analysis. 6<sup>th</sup> International Workshop on the Biology of Fish Gametes, Vodnany, Czech Republic, 4-7 September 2017, book of abstracts p. 91.

**Kollár, T., Kása, E., Csenki-Bakos, Z., Ferincz, Á., Urbányi, B., Horváth, Á.:** Development of an *in vitro* toxicological test system based on zebrafish (*Danio rerio*) sperm analysis. 10<sup>th</sup> European Zebrafish Meeting, Budapest, Hungary, 3-7 July 2017, book of abstracts p. 369.

**Kollár, T., Kása, E., Ferincz, Á., Urbányi, B., Csenki-Bakos, Z., Horváth, Á.:** Toxicological test system based on zebrafish (*Danio rerio*) sperm analysis. International Conference of the Hungarian Laboratory Animal Association, Budapest, Hungary, 25 May 2017.



**Kollár, T., Kása, E., Ferincz, Á., Urbányi, B., Csenki-Bakos, Z., Horváth, Á.:** Development of toxicological test systems based on fish sperm analysis. XIV. PhD – Conference organized by Professors for European Hungary Association, Budapest, Hungary, 6 April 2017, book of abstracts p. 29-35.

**Kollár, T., Csenki, Z., Kovács, R., Bernáth, G., Horváth, Á.:** Effect of different fluorides on the quality of zebrafish (*Danio rerio*) sperm. 20<sup>th</sup> Youth Scientific Forum” international conference, Keszthely, Hungary, 23-24 May 2014, book of abstracts p. 38-47.

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### **Publications in scientific journals:**

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