BIOLOGIJA. 2007. Vol. 53. No. 3. P. 1–5

Evaluation of DNA damage by means of the comet assay and micronucleus test in erythrocytes of Prussian carp (*Carassius auratus gibelio*) infected with ulcerative disease

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The influence of ulcerative disease infection on the level of DNA damage was assessed in erythrocytes of Prussian carp (*Carassius auratus gibelio*). Microbiological examination of the experimental fish revealed the presence of *Aeromonas* and *Pseudomonas* bacteria in infected fish. Ten blood samplings were performed with the same fish individuals before and after i. p. injections of antibiotic oxytetracycline (OTC). The comet tail moment (CTM), the frequencies of micronucleated (MN) and amitotic (AM) erythrocytes were evaluated. The level of CTM in infected fish was higher than in controls ($p < 0.0001$) and significantly decreased after OTC injections ($p < 0.0001$). The frequency of MN erythrocytes was systematically higher in infected fish ($p = 0.003$) and did not decrease after OTC injections ($p = 0.918$). Thus, infection triggered DNA damage in fish erythrocytes. The levels of amitotically dividing erythrocytes were elevated in all individuals, and there were no significant difference between the infected and control groups ($p = 0.8$). Probably both infection and repeated blood sampling were the reasons for amitotic division of erythrocytes.

Key words: micronuclei, comet assay, genotoxicity, amitosis, erythrocytes, bacterial infection, *Carassius auratus gibelio*

INTRODUCTION

The group of separate diseases with similar clinical symptoms and pathogenic alterations comprises carp spring virusemia, erythrodermatitis, aeromonosis, pseudomonosis [1]. Regarding skin ulcerations, these diseases may be characterized as ulcerative disease or ulcerative disease syndrome [2, 3]. Factors like fish overcrowding, stress, oxygen deficiency and high temperature may cause disease outbreak [4]. Fish skin abrasions are the "opened gates" for pathogenic bacteria such as *Aeromonas hydrophila* [5]. Depending on the severity of infection, fish exhibit a variety of external and internal pathological changes [6]. Such fish are an interesting object for various scientific research, including identification of pathogenic bacteria and assessing their resistance to antibiotics [7], monitoring of hematological and biochemical parameters in infected and treated fish [5], for a variety of immunological studies [8, 9]. However, little is known about the level of DNA damage in fish during infection. There are some studies showing the ability of certain viruses and bacteria to induce mutations and cause cancer in humans [10–13]. It might be that pathogens such as viruses and bacteria are also capable of inducing DNA damage in the fish organism and thus could be characterized as a potential genotoxic factor in an aquatic environment. Therefore, it was interesting to elucidate DNA damage in fish infected with ulcerative disease. During this study, the micronucleus (MN) test and the comet assay were used to evaluate DNA damage in erythrocytes of Prussian carp (*Carassius auratus gibelio*).

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MATERIALS AND METHODS

Fish

Two Prussian carps (*Carassius auratus gibelio*) weighing 83 g and 78 g, with visible ulcers on the skin were obtained from a supermarket (Infected 1 and Infected 2), and three control fish (Control 1–3) came from aquaculture (65 g, 59 g and 55 g). The fish were acclimated for 12 days in two separate 25 l aquariums (infected group and control) and fed every day with dried gammarides. Throughout the experiment, water temperature was kept stabile (18 °C).

Experimental design

Blood samples were taken with a syringe from each fish for the comet assay and micronucleus test. Due to the low number of individuals under study, blood sampling was performed repeatedly every 10 days to obtain a sufficient data sample for statistical analysis. Infected fish were subjected daily to 10 μg/kg oxytetracycline (OTC) i. p. injections for 10 days after the fifth sampling. The same injections were performed for two control fish (Control 1 and Control 2) after the 10th blood sampling.

Comet assay

The single-cell gel electrophoresis assay was performed according to Gichner et al. [14] with some minor changes. Freshly withdrawn blood (6 µl) was diluted in 1 ml of cold PBS solution (pH 7.5). 40 µl of blood cell suspension was mixed with 40 µl of 1% low melting point (LMP) agarose gel at 37 °C on a microscope slide pre-coated with a thin layer of 1% normal melting point (NMP) agarose gel. The slide was coated with a cover slip and put on ice for no longer than 5 min to allow the gel to solidify. The cover slip was then removed and a third layer of 80 µl of 0.5% LMP agarose gel was applied. The slide was covered with a cover slip again and left on ice for 5 min for solidification. After the agarose gel had solidified, the cover slips were removed and slides were placed in freshly made lysis solution (2.5 M NaCl, 100 mM Na_2 EDTA, 10 mM Tris, pH 10, 10% DMSO and 1% Triton X-100) at 4 °C overnight. After lysis, the slides were placed on a horizontal gel electrophoresis tray containing freshly prepared electrophoresis buffer (300 mM NaOH, 1 mM Na_2 EDTA, pH > 13) for 20 min to allow DNA unwinding. Electrophoresis was then carried out at 20 V, 300 mA for 20 min. Thereafter, the slides were neutralized with Tris-HCl (pH 7.5) for 15 min, stained with 70 µl of 20 µg/ml ethidium bromide for 5 min, washed with distilled water (5 min) and covered with cover slips. Slides were examined at 600× magnification with an Olympus Provis fluorescence microscope (420–490 nm excitation filter and a 520 nm emission filter), equipped with a Hamamatsu C4742-95 digital camera. Comet images were obtained using Micro Image 4.0 software. At least 100 comet images per slide were analysed in TIF format with CASP 1.2.2. program [15]. Comet tail moment (CTM) – the tail length multiplied by the percent tail DNA – was chosen as a parameter representing the degree of DNA damage in erythrocytes.

Micronucleus test

A drop of blood from the caudal vessel was taken with a heparinised syringe, smeared on slides and air-dried. After fixation in methanol for 10 min, the slides were stained with 10% Giemsa solution for 8 min, rinsed with distilled water and dried.

The frequency of micronucleated and amitotic erythrocytes was evaluated by scoring at a 1250 × magnification. A total of 10 000 mononucleated erythrocytes were examined for each fish. Only cells with the intact cellular and nuclear membrane were scored. Round or ovoid-shaped non-refractory particles with the colour and structure similar to chromatin, with a diameter 1/3–1/50 of the main nucleus and clearly detached from it were interpreted as micronuclei. The eight-shaped outstretched erythrocytes formed by the constriction of the nucleus into two new nuclei of equal or unequal size were interpreted as amitotically dividing.

Statistical analysis

All experimental data were processed with SPSS 10.0 statistical software. One-way ANOVA (analysis of variance) was performed to estimate differences between infected and control fish and the effect of oxytetracycline injections. All data are presented as mean ± S.E.M. (standard error of the mean). Statistically significant difference was set as $p < 0.05$.

RESULTS

Comet assay

The comet tail moment (CTM) of erythrocytes was estimated for each fish in every subsequent experiment. The total levels of CTM of each fish and the effect of OTC are shown in Fig. 1. Analysis of variance of CTM revealed significant differences between infected and control fish ($p < 0.0001$) and differences in CTM values in infected fish before and after treatment with antibiotic OTC ($p < 0.0001$). The CTM of infected fish were obviously higher than those of control fish and after OTC injections notably decreased. It may be concluded that after the last tenth sampling the CTM values of OTC-treated fish decreased to the control level ($F = 1.514$, $p = 0.219$). Two control fish (Control 1 and Control 2) were also injected for 10 days with OTC after the set of 10 blood samplings had been finished. After the treatment, the low values of CTM decreased even more – from 0.14 and 0.60 to 0.05 and 0.14, respectively ($F = 26.009$, $p < 0.0001$).

Micronucleus test

The frequencies of micronucleated (MN) and amitotic (AM) erythrocytes were estimated for each fish in every subsequent experiment. The total levels of MN and AM of each fish and the effect of OTC injections are shown in Figs. 2 and 3. Analysis of variance of MN revealed significant differences between infected and control fish ($p = 0.003$) while OTC injections did not affect the frequency of micronucleated erythrocytes ($p = 0.918$). The levels of amitotically dividing erythrocytes significantly differed between individuals ($p < 0.0001$) but did not differ between infected and control group ($p = 0.800$) and were not influenced by OTC injections ($p = 0.322$). A low but significant correlation was found between CTM and MN ($r = 0.288$, $p = 0.042$).

Microbiological examination

After finishing the experiments, fish were examined for the presence of pathogenic bacteria at the National Veterinary Laboratory (Vilnius, Lithuania). The results are presented in Table. A dominant species of bacteria *Aeromonas hydrophila* was found in the heart and liver of both infected fish and in kidneys of the Infected 1 fish. Also, in *Aeromonas hydrophila* bacteria were found in Control 1 and *Pseudomonas cepacea* in Control 2. Examination of fish for the presence of carp spring virusemia showed negative results.

DISCUSSION

Fish are in an intimate contact with their environment which contains high concentrations of viruses and bacteria. Fish defend themselves by a great variety of methods, including non-specific and specific immune response mechanisms [8, 16]. At the same time pathogenic viruses and bacteria try to escape host defense. During infection, *Aeromonas hydrophila* releases a variety of endo- and exotoxins (haemolysins, enterotoxins, aerolysins, cytotoxins, leucocidins) and induces apoptosis in *Carassius auratus* lymphocytes *in vitro* [4]. In our study, very high values of CTM in erythrocytes and elevated levels of MN were found in infected fish. Moreover, *A. hydrophila* was found in the hearts of both infected fish, which indicated the presence of these

Table. **Results of microbiological examination of infected and control fish**

Individual	Ulcer on operculum	Heart	Liver	Kidneys	Brain
Infected 1	*Pseudomonas maltophila*	*Aeromonas hydrophila, Pseudomonas cepacia*	*Aeromonas hydrophila*	*Aeromonas hydrophila*	Not found
Infected 2	–	*Aeromonas hydrophila*	*Aeromonas hydrophila*	Not found	Not found
Control 1	–	Not found	*Aeromonas hydrophila*	Not found	Not found
Control 2	–	Not found	*Pseudomonas cepacia*	Not found	Not found
Control 3	–	Not found	Not found	Not found	Not found

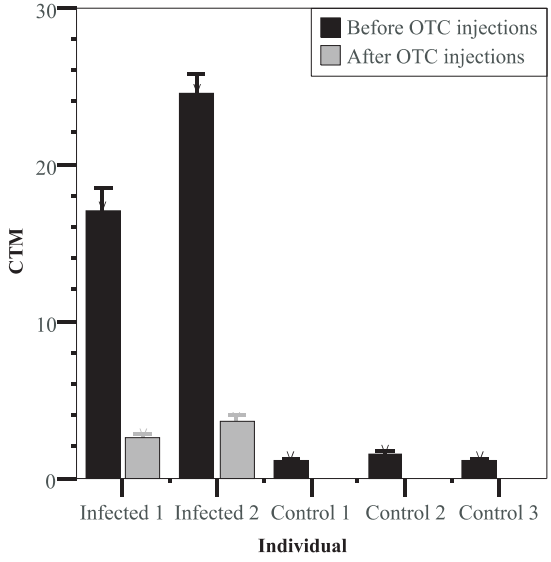

Fig. 1. Total levels of comet tail moment (CTM) of each fish and the effect of OTC injections (bar represents mean ± S.E.M.). Ten repeated blood samplings were performed from each fish. For infected fish, pooled results of five blood samplings before and five after OTC i. p. injections (10 µg/kg daily for 10 days) are presented. Control fish were not treated with OTC during this set of samplings, therefore summed results of 10 samplings are shown

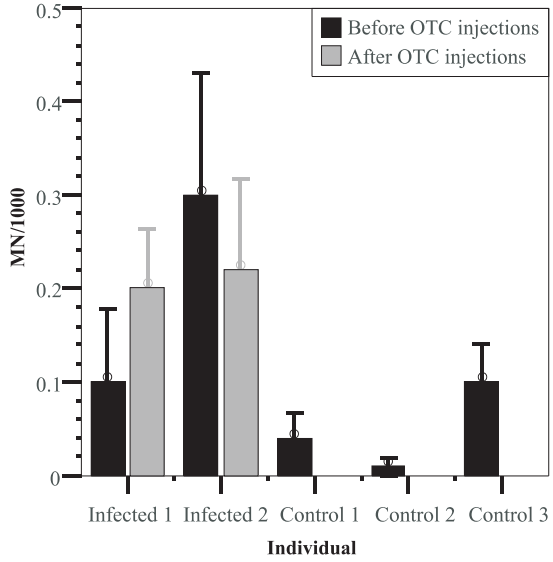

Fig. 2. Total frequency of micronucleated (MN) erythrocytes of each fish and the effect of OTC injections (bar represents mean ± S.E.M.). Ten repeated blood samplings were performed from each fish. For infected fish, pooled results of five blood samplings before and five after OTC i. p. injections (10 µg/kg daily for 10 days) are presented. Control fish were not treated with OTC during this set of samplings, therefore summed results of 10 samplings are shown

pathogenic bacteria in the fish bloodstream. It might be that exotoxins and other metabolites of these bacteria could cause DNA damage in erythrocytes. Basheera et al. [17] determined an increased production of superoxide anion by head-kidney leucocytes of Indian major carp immunized with *Aeromonas hydrophila,* while Tanaka et al. [18] elucidated accumulation of hydroxy lipids in the muscles and liver of fish infected with various pathogenic bacteria. There are data from human studies showing that all carcinogenesis via microbial infection involves free radical generation, furthermore DNA strandbreaks are frequently observed [19]. Various infections, including bacterium *Helicobacter pylori,* hepatitis B and C viruses, Epstein–Barr (EB) virus, some parasites (*Opisthorchis viverrini* and *Schistosoma mansoni*) have been suggested to be risk factors of hepatoma, gastric and other types of cancer, and the cause may be closely related to the formation of free radicals and reactive nitrogen oxides [20]. These findings lead us to the assumption that DNA damage in erythrocytes of infected fish could be caused by reactive oxygen species formed during infection. After OTC injections we observed partial or complete healing of wounds on the infected fish surface and decreased levels of CTM. Therefore we can conclude that OTC slowed down the growth of pathogenic bacteria and strengthened the immunity of fish.

The frequency of micronucleated erythrocytes was systematically higher in infected fish than in controls, but there were no differences before and after OTC injections. This suggests that infection-affected hemopoietic organs produce more micronucleated cells. Interestingly, Ning et al. [21] also observed increased levels of micronuclei in *Helicobacter pylori* infected human gastric mucous epithelian membrane.

There were also numerous AM erythrocytes found in both infected and control fish. Blood insufficiency or anemia could induce amitosis in erythrocytes. Most likely the level of AM erythrocytes increased because of repeated blood sampling during experiments. Nevertheless, it is unclear why there were practically no amitotically dividing erythrocytes in the control fish No. 2. The impact of infection on the level of AM could be assessed by analysing the results of the first blood sampling when the volume of blood was not affected by repeated blood sampling. The level of AM in the infected fish No. 1 and No. 2 during the first experiment was 0.1‰ and 4.0‰, respectively and there were no AM in control fish. Therefore, probably both factors, blood sampling and infection, contributed to the raised level of AM erythrocytes.

This study was carried out with the aim to investigate the relationship between fish infection and the level of DNA

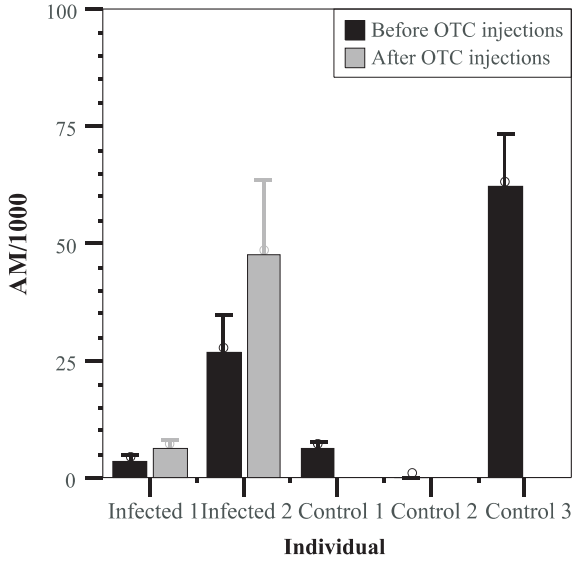

Fig. 3. Total frequency of amitotic (AM) erythrocytes of each fish and the effect of OTC injections (bar represents mean ± S.E.M.). Ten repeated blood samplings were performed from each fish. For infected fish, pooled results of five blood samplings before and five after OTC i. p. injections (10 µg/kg daily for 10 days) are presented. Control fish were not treated with OTC during this set of samplings, therefore summed results of 10 samplings are shown

damage in erythrocytes by means of the MN test and comet assay. Both these methods have been widely used jointly or separately in a variety of genotoxicological studies using fish as a test organism [22–26]. There are some controversial data concerning the applicability of these methods for *in situ* studies. Russo et al. [26] assessed the genotoxicity of pollutants of the Sarno River for erythrocytes of *Gambusia holbrooki* and pointed out the suitability and sensitivity of the MN test and comet assay. On the contrary, Bombail and Aw [27] applied these two methods to butterfish (*Pholis gunnellus*) erythrocytes from the Firth of Forth, Scotland, and had some doubts about suitability of the comet assay for *in situ* studies. Moreover, the results of our study have clarified the effect of infection on the level of DNA damage in fish erythrocytes. There is a relation between pollution and fish susceptibility to various diseases. It is known that various PCBs, PAHs and heavy metals suppress the immune system of fish and make them more susceptible to infections [28, 29]. On the other hand, infected fish may be more susceptible to intoxication since the parasites reduce resistance to all stress factors, poisons included [30]. According to our results, infection also induces DNA damage. Both the comet assay and micronucleus test are non-specific for mutagenic damage, thus it is possible to obtain false-positive results when assaying the genotoxicity of pollutants *in situ*. For this reason we recommend examining fish health state (i. e. the presence of pathogenic bacteria, viruses or parasites) when performing genotoxicological studies *in situ*.

Received 1 February 2007
Accepted 19 June 2007

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RAUDONLIGE SERGANČIŲ SIDABRINIŲ KAROSŲ (*CARASSIUS AURATUS GIBELIO*) DNR PAŽAIDŲ ERITROCITUOSE NUSTATYMAS KOMETOS IR MIKROBRANDUOLIŲ METODAIS

Santrauka

Kometos ir mikrobranduolių metodais įvertintos DNR pažaidos raudonlige sergančių karosų (*Carassius auratus gibelio*) eritrocituose. Dešimties pakartotinai atliktų eksperimentų metu buvo matuojamas kiekvieno karoso eritrocitų kometos uodegos momentas (KUM), įvertinti eritrocitų su mikrobranduoliais (EMB) ir amitozinių (AM) eritrocitų dažniai. Atlikus duomenų dispersinę analizę (ANOVA) nustatyti statistiškai patikimi KUM reikšmių skirtumai tarp ligotų ir kontrolinių žuvų, taip pat KUM reikšmių skirtumai iki ir po antibiotiko oksitetraciklino (OTC) injekcijų ligotoms žuvims ($p < 0,0001$). Ligotų karosų EMB dažniai statistiškai patikimai aukštesni nei kontrolinių žuvų ($p = 0,003$), o OTC injekcijos neturėjo įtakos EMB dažniui ($p = 0,918$). Amitozinių eritrocitų lygis buvo pakilęs tiek ligotų, tiek kontrolinių žuvų, bet patikimo skirtumo tarp šių dviejų grupių nebuvo ($p = 0,8$). Atlikus mikrobiologinius tyrimus nustatytos *Aeromonas* ir *Pseudomonas* genčių bakterijos sergančių žuvų širdyse.