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RESEARCH ARTICLE

PRELIMINARY STUDY ON DETERMINATION OF DNA DAMAGE OF *Cyprinus carpio* BY SINGLE CELL GEL ELECTROPHORESIS IN IŞIKLI LAKE, DENIZLI

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ABSTRACT

In this study, DNA damage was examined in blood samples taken from *Cyprinus carpio* between October 2019 and December 2019. The aim of this study is to determine single strand breaks in DNA by single cell gel electrophoresis (comet analysis). Different concentrations of Methyl methanesulfonate (MMS) was applied *in vitro* conditions and pollution of lake was evaluated on a cell basis. DNA damage was found to be less than the concentrations of MMS applied in October 2019. In December 2019, DNA damage was found to be less than on the basis of cells compared to different concentrations of MMS. Average Arbitrary Units (AAU) was calculated. Accordingly, the samples taken in October 2019 AAU values were obtained 1,4044±0,2725, after MMS application the results were between 2,38±1,1637 and 2,824444±0,6556. And in December 2019, AAU values in blood samples were found to be between 298278±0,2213, and MMS values between 2,35±0,8381 and 2,884167±0,657. Comet head and tail lengths were calculated from the comet images obtained from the comet parameters. Comet tail lengths were determined as 15.784 ± 6.97 µm in October 2019 and 10.899 ± 4.75 µm in December 2019. The data obtained from results, there is not much DNA damage of *C. carpio* in Işıklı Lake. As a result *Cyprinus carpio* is an efficient organism to evaluate genotoxicity of environmental pollution and the pollution in Işıklı Lake is not at critical level.

Keywords*: DNA damage, Single cell gel electrophoresis, Işıklı Lake, Cyprinus carpio.*

1. INTRODUCTION

The environment is continuously loaded with exogenous and endogenous substances released by anthropogenic activities and, this effect directly or indirectly the genetic material of living organisms. Environmental pollution by the deposition of contaminants during a long time causes an increase in the aquatic habitat. The pollutants can cause damage on the aquatic organisms' genetic material, and this can pass onto the next generation. [1]. Endogenous and exogenous changes that affect the molecular integrity of living things are called "DNA damage" [2]. When the DNA cannot be repaired, genomic instability occurs, which is important for the future of the organism [3].

Aquatic organisms accumulate pollutants directly from contaminated water and indirectly by feeding on contaminated organisms. Fish populations are decreasing due to increase in industrialization which causes destruction of habitats with adding mutagenic and genotoxic chemicals to wetland ecosystems. [4,5]. In addition, it produces effective chemical products such as pesticide, heavy metals, food additives, industrial residues, radiation, relative oxygen species (ROS) in the organism. These products interact with macromolecules such as DNA, lipid and protein in the cells of the organism, leading to DNA damage, mutagenicity, carcinogenicity and aging [5,6].

Detection of DNA damage is provided by biological monitoring tests. The comet assay is a suitable technique to measure various types of DNA damage. This test used in genotoxicology, detects single strand breaks in DNA by single cell gel electrophoresis, which is a fast, reliable method [7]. This method was applied for the first time by Ostling and Johansenn (1984), and Singh et al. (1988) and named as single cell electrophoresis (Comet analysis) [8,9]. DNA damage is quantified by the proportion of DNA which migrates out of the nuclei toward the anode when individual cells or isolated nuclei, embedded in a thin agarose laye are subjected to electrophoresis that results on a comet-like shape of nuclei. This enables quantification of DNA in comet tails after staining with ethidium bromide. The longer the comet's tail observed means the more single strand breaks in DNA which points the genetic material of the organism is damaged for various reasons. Undamaged DNA appears as a tailed ring.

Işıklı Lake is located in the upper basin of the Büyük Menderes River, south of Akdağ, with a maximum surface area of approximately 64.00 km^2 within the borders of Denizli province, in Çivril district [10,11,12]. The lake is fed by Akçay Stream, Kufi Stream, Işıklı Founts, two large tributaries in the upper basin of Büyük Menderes and groundwater at the bottom. There are reed islands in the middle of the lake [10,12,13]. The lake water is used for agricultural irrigation. Işıklı Lake is a natural lake with the floods where the water level rises. In order to protect the agricultural areas and settlements, the State Hydraulic Works (DSI) made a dam and turned into a dam lake [10,12,14].

In this study, we described the application of the comet assay to nuclei of *Cyprinus carpio*. The purpose of the study was to evaluate the induction of DNA damage in this fish species. For that purpose Methyl methanesulfonate (MMS), a genotoxic chemical causing DNA breaks was used.

2. MATERIAL AND METHODS

2.1. Material

Carp fish *(Cyprinus carpio)* blood samples were taken from two different stations (38°15'33.8"N 29°55'10.0"E and 38°15'06.8"N 29°53'26.8"E) from the local fishermen and stored in the laboratory.

2.2. Chemicals

Dulbecco's Phosphate-Buffered Saline (DPBS) (PanBiotech), Tris (BioShop, CAS 77-86-1), Triton X-100 (BioShop, CAS 9002-93-1), N-Laurylsarcosine-sodium salt (Sigma-Aldrich Co, CAS 137-16-6), Methyl methanesulfonate (MMS) (Sigma Aldrich, CAS 56-27-3), Ethidium Bromide (BioShop, CAS 239-45-8), Normal melting agarose (NMA) (Amresco,), Low melting agarose (LMA) (Sigma-Aldrich, CAS 9012-36-6).

2.3. Method

2.3.1. In vitro positive control application in cells

Methyl methanesulfonate (MMS) concentration of 60uM, 70uM and 80uM were used to trigger DNA damage, respectively. DNA was exposed to MMS for 15 minutes at 0^{o}C and washed with DPBS.

2.3.2. Single cell gel electrophoresis (Comet assay)

The Comet assay protocol was performed with minor modifications from the literature [15]. Blood samples taken from the species were washed with DPBS and mixed with 0.5% low melting point agarose. After taking 10ul of this mixture, the slide covered with 1% normal melting point agarose was spread and the agarose was frost by covering the coverslip (10 min, 4^{o}C). The coverslips on the slides were removed and placed in cold lysis solution (2,5M NaCl, 100mM EDTA, 10mM Tris, (%10 DMSO, %1 Triton X-100, %1 N-Laurylsarcosine-sodium salt pH 10) for 1 hour at 4^{o}C. Slides were placed in a gel-electrophoresis filled with electrophoresis buffer (10M NaOH, 5M EDTA, pH 13) for 20 minutes. Electrophoresis was performed in the same buffer and run for 20 minutes at 25V, 300mA. After electrophoresis, slides were neutralized with the solution (pH 7.5) for 5 minutes. Each slide was stained with Ethidium bromide (20ul/ml) for 20 minutes and examined under a fluorescent microscope (Nikon ECLIPSE 50i) in a suitable barrier filter light. The CometScore2.0.0.38 program was used to distinguish the cells visually and ranges them from 0 to 4 to assess the extent of the contamination. In addition, head and tail lengths were calculated from the comet parameters of 20 selected comet.

2.3.3. Statistical analysis

SPSS V25.0 package program was used for statistical analysis of the data. The normality of the data was evaluated with the Shapiro-Wilk test. After the groups that did not show normal distribution in the comet categories, Kruskal-Wallis test was used for the comparison ($p>0.05$). The average of cell numbers obtained from each sample, their standard deviations were calculated and Arbitrary Units (AU) evaluation was made at $p> 0.05$ significance level. The measurements of the tail and head parts of the comets were made with the Image J program. The Shapiro-Wilk test was applied to determine the distribution of the data. Normally distributed data were evaluated with Student-T test with $p> 0.05$ significance level.

3. RESULTS

In this study, single cell gel electrophoresis (comet analysis) was applied to blood samples taken from *Cyprinus carpio* in October 2019 and December 2019. The results of comet damage levels from the samples taken in October 2019 was found to be 0 degrees and meaning the genetic damage was not very high. Methyl methanesulfonate (MMS) was applied to cells at 60uM, 70uM and 80uM concentrations (Fig. 1). Previous studies have shown that MMS causes DNA-strand breaks. In this study we also observed that higher MMS concentrations show higher DNA damage. A decrease was observed between samples that received 4th degree damage and the control groups.

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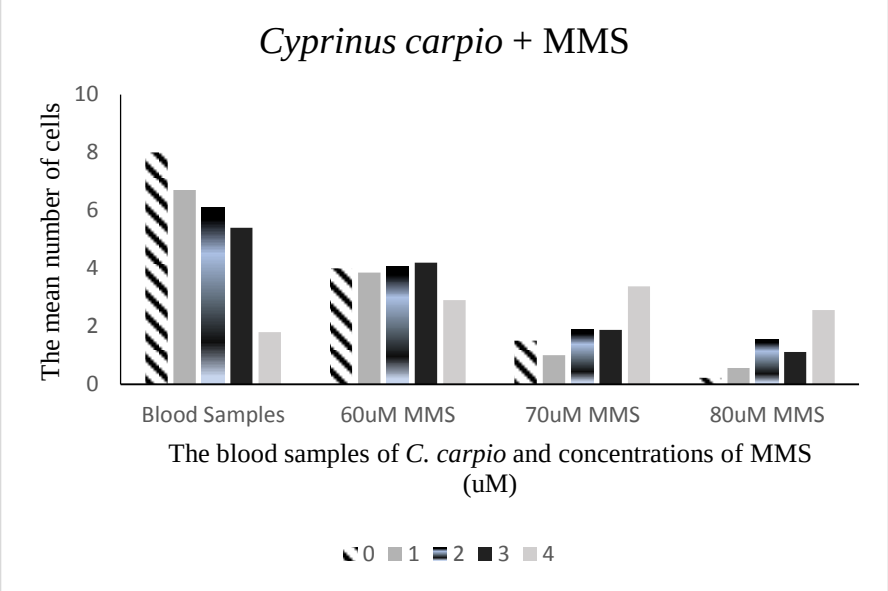

Figure 1. Comparisons between the DNA damage levels of samples (October 2019)

The comet categories of the samples (December 2019) and the comet categories of MMS-induced samples are given in Figure 2. A decrease is observed when blood concentrations of 0 degree are compared with 60uM, 70uM and 80uM concentrations of MMS. Compared to cells containing damaged DNA of grade 4, an increase was observed between blood samples and MMS concentrations applied at 60uM, 70uM and 80uM and this increase caused less damage to the 60uM MMS concentration (Figure 2).

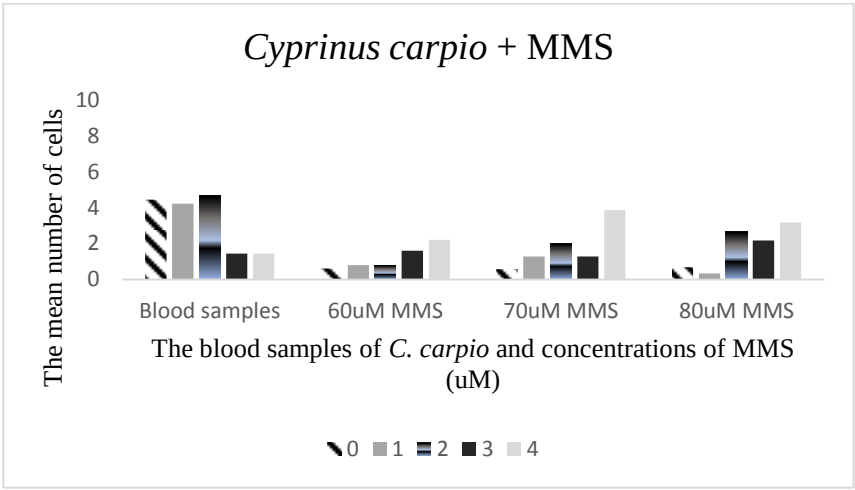

Figure 2. Comparisons between the DNA damage levels of samples (December 2019).

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Arbitrary units were calculated by classifying and counting the cells. To calculate Arbitrary units: 0xA + 1xB + 2xC + 3xD + 4xE / N where 0, 1, 2, 3 and 4 comet categories, A, B, C, D and E are the number of cells counted in these categories and N represents the total number of cells [16]. The average arbitrary unit data of the blood samples and the samples exposed to MMS are given in Table 1. There is an increase in average Arbitrary units (AAU) values in blood samples compared to positive controls. The results support that the fish species from the lake has less DNA damage than the MMS induced samples. Cells were classified according to comet tail length. Class A has no tail and spotted, class B has a small tail head (nucleus) diameter, class C has a tail longer than class B head diameter, class D has a tail longer than class C head diameter, class E has the longest comet tail (apoptotic cells) (Figure 3).

Table 1. Average arbitrary units (AAU) values (±standard deviation). (Data were evaluated at p>0,05 significant difference).

October 2019 Samples	**Average Arbitrary Units (AAU)**	**December 2019 Samples**	**Average Arbitrary Units (AAU)**
Without MMS	1,4044±0,2725	Without MMS	1,298278±0,2213
60uM MMS	2,38±1,1637	60uM MMS	2,35±0,8381
70uM MMS	2,341563±0,7777	70uM MMS	2,937857±0,696
80uM MMS	2,824444±0,6556	80uM MMS	2,884167±0,657

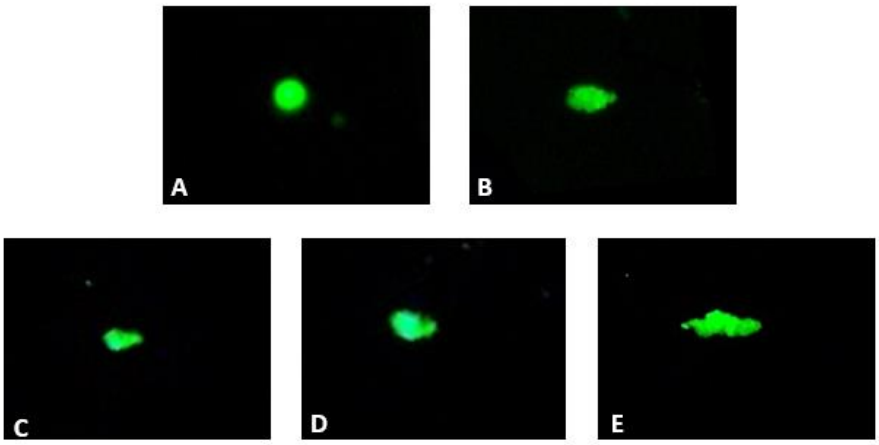

Figure 3. Comets categories of Comet assay, A, undamaged DNA, B, C, D damaged DNA, E very damaged DNA.

The percentages of the observed cells separared by comet categories (Table 2). According to the results, comets of 0 degree in the blood samples without MMS were observed more than other samples in December 2019. It is observed that in the MMS induced samples, 43% of the 4th degree comets were in the range of 70uM MMS in December 2019 and 80uM MMS in October 2019. The results prove the

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DNA damage of 70uM MMS. When the fish samples taken from the lake are compared with the ones treated with MMS, it is seen that the lake is not damaged more than MMS induced samples.

Table 2. Comet categories and their percentages of *Cyprinus carpio* from Işıklı Lake-Turkey.

Comet Categories						
					Seasons	
0	**1**	**2**	**3**	**4**		
26%	30%	23%	13%	7%	October 2019	**Without MMS**
36,13%	18%	25%	13%	8%	December 2019	
27%	21%	12%	18%	22%	October 2019	**60uM MMS**
10%	13%	13%	27%	37%	December 2019	
16%	10%	19%	19%	35%	October 2019	**70uM MMS**
6%	14%	22%	14%	43%	December 2019	
4%	9%	26%	19%	43%	October 2019	**80uM MMS**
7%	4%	30%	24%	35%	December 2019	

Table 3. Results of the tail length and head length of comets (±standard deviation). (Data were evaluated at p>0,05 significant difference).

		The tail lenght of comets (µm)		**The head lenght of comets (µm)**	
	N	**October 2019 Samples**	**December 2019 Samples**	**October 2019 Samples**	**December 2019 Samples**
Without MMS	20	15,784±6,97	10,899±4,75	7,484±2,60	5,138±1,81
60uM MMS	10	36,131±15,27	27,115±10,56	12,078±5,92	16,461±3,48
70uM MMS	10	30,781±9,22	22,088±6,72	19,249±6,55	21,282±8,22
80uM MMS	10	37,277±20,20	41,201±18,58	21,027±8,99	22,932±7,77

In this study, no significant difference was found between 10.899 ± 4.75 µm and 15.784 ± 6.97 µm in the comet tail length analyzes (p> 0.05). The comet queue lengths of the MMS applied samples are approximately 2 times longer than the lake samples. Comparing comet tail lengths between October and

December, the comet tail length in October 2019 is approximately 1.5 times longer. When comet head lengths were examined, the values were evaluated as 7.484 ± 2.60 µm and 5.138 ± 1.81 µm ($p > 0.05$). Comet head lengths are longer in the samples with MMS than MMS (-) samples (Table 3). The comet head length in the samples in October 2019 occurred more than the December 2019 samples, and the difference between them was approximately 0.5 times. without MMS (Table 3). This length is approximately 0.5 times longer in MMS induced samples. The comet head length in the samples in October 2019 occurred more than December 2019 samples, and the difference between them was approximately 0.5 times. The highest tail length was found at 80uM MMS concentration in December 2019 and comet head length at 80uM MMS concentration in December 2019.

4. DISCUSSION AND CONCLUSION

In this study, degree of DNA damage of *C. carpio* from Işıklı Lake, *in vitro* conditions were evaluated by single cell gel electrophoresis (comet assay). The mutagenic effect of whole blood cells exposed to Methyl methanesulfonate (MMS) was examined as a positive control. The pollution status of the lake was evaluated by comparing the blood samples of the fish taken from the lake and at the different concentrations of MMS. Considering the Comet percentages, the results showed that DNA damage occurred in December 2019 was the highest. When samples were induced to MMS, the most DNA damage was in December 2019 samples compared to October 2019 samples (Table 2). According to MMS concentration of DNA damage, in October 2019 samples was less than in December 2019 samples. When the average arbitrary units values were examined, for October 2019 samples it is 1.4044 ± 0.2725 and 1.298278 ± 0.2213 for December 2019 samples were determined (samples without MMS) (Table 2). After the MMS induction, values incresed to 2.38 ± 1.1637 for October 2019 samples and 2.35 ± 0.8381 for December 2019 samples. For the samples that were induced to MMS, we can say that the DNA damage has increased gradually as there is an increase in OAU values. The highest increase occurred as 70uM MMS from the December 2019 samples. The tail and head lengths of the comets were calculated from the comet parameters. According to these calculations, DNA damage increases as the tail and head length of the comets increase.

Methyl methanesulfonate (MMS) is an alkylating agent that causes DNA damage [17]. In this study, an increase was observed in cells damaged by the 4th degree. Kammann et al. (1999), applied comet analysis of leucocyte samples of *C. carpio* from the North Sea using various concentrations and various terms of H_2O_2 as a positive control in their study. They emphasized that organic sediment has a genotoxic potential and the comet technique is a powerful technique detecting DNA breaks [18]. Rank and Jensen (2003) applied comet assay on the gill cells and hemocytes of *Mytilus edulis* with exposed to MMS *in vitro* and *in vivo* conditions. As a result, the cells exposed to MMS did not show toxicological effects even at high concentrations [19]. Lemos et al., (2005) performed a comet analysis on *Tilapia rendalli*, and the number of scores in the MMS applied cells was higher than the score number of the control groups. Kammann et al., (1999) found the lowest result from arbitrary unit points as 4, which is 2 times higher than the Işıklı Lake. Besides this, Lemos et al. (2005) DNA damage results were 13 times more than the Işıklı Lake, and 16 times more DNA damage was found in the MMS induced samples.

Çok et al. (2011) detected DNA damage on *C. carpio* from Lake Mogan using with Comet assay method. And they found the length of the comet tail as 31.10 ± 10.39 µm. According to results of their study we can say that there is more DNA damage in fish samples in Lake Mogan than the samples from our study. [21]. Kontaş and Bostancı (2020), conducted heavy metal analyzes and genotoxic

investigations on *Capoeta banarescui* taken from Melet River and the tail lengths of comets in autumn season were determined as 20.47 ± 1.30 µm. Compared to Işık Lake, it has approximately 1.5 times longer tail length, and this indicates that Melet River suffers more DNA damage than Işık Lake [22]. On the other hand, Arslan et al., (2016) found that comet tail lengths were approximately similar to Lake Işıklı in a study conducted with comet assay on *Oncorhynchus mykiss* in Pınarbaşı district of Kayseri [23].

Single cell gel electrophoresis has a very fast, sensitive and common protocol [24]. This method, also known as comet analysis, is often used in ecotoxicology to detect the genotoxic effect of some chemical or physical agents on the model organism [3,25,26]. The mutagenic effect of Methyl methanesulfonate (MMS) used in the research is evident in the results and has played a role in the DNA damage as a direct alkylating agent.

The comet assay is now widely used to measure DNA damage and it is becoming a primary tool for pollutant biomonitoring in the aquatic environment and some authors suggest that the comet assay is more sensitive than the other genotoxicity tests [27,28,29]. In conclusion, the pollution in Işıklı Lake was not worrying and *Cyprinus carpio* is an efficient organism to evaluate genotoxicity of environmental pollution, they are sensitivity to pollutants, especially in comet assay. However, these studies are the preliminary studies about the pollution of Işıklı Lake and further work is needed, including analyzing the DNA damage in fish tissues, ecotoxicological studies of water and sediment samples.

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