Comet and Micronucleus Assays for Detecting Benzo (a) Pyrene Genotoxicity in Blood Cells of Nile Tilapia (Oreochromis Niloticus) from the Shatt Al-Arab River in Southern Iraq

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Abstract

On days 0, 1, 3, 6, and 12, the proportion of DNA strand breakage (determined using the comet test) and micronucleus formation in blood cells were observed in Nile tilapia (*O. niloticus*) exposed to benzo(a)pyrene at doses of 0, 15, and 30 µg/L. The comet test results showed that the proportion of strand breaks increased with increasing concentrations of benzo(a)pyrene, but levels of DNA damage decreased significantly after 12 days of exposure at all tested concentrations, indicating that the patterns of changes in DNA breakage levels can be explained by the threshold-dependent DNA repair theory. Furthermore, the relatively sluggish development of the DNA damage response and recovery in Nile tilapia blood cells in comparison to prior research utilizing *O. niloticus* livers suggests that the DNA modification response to benzo (a) pyrene exposure in this species is tissue-specific. Monitoring the frequency of micronucleus formation in fish blood cells during the exposure period revealed a dose-time-response relationship. The significant association between micronucleus induction and DNA strand breaking suggests a probable cause-and-effect relationship between the two types of damage. We propose that genotoxin exposure in the marine environment can be detected by DNA strand breakage and micronucleus formation in fish blood cells.

Keywords: Nile Tilapia, Benzo(a)Pyrene, Blood Cells, DNA Breakage, Comet Test, Micronuclei.

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INTRODUCTION

Persistent organic pollutants are substances with a nearly human origin that are expected to be resistant to degradation by photolysis, chemical change, and/or biological activity (Ashraf, 2017). Because of their toxicity and endurance, persistent organic pollutants pose a serious threat to all living things. Due to their environmental persistence, many species may be exposed to low amounts of persistent organic pollutants over an extended period of time. As a result, major chronic impacts may emerge in a range of cell types, affecting the entire organism (Gonzalez-Mille *et al*., 2010). Because of bioaccumulation in aquatic ecosystems, where the concentration of persistent organic pollutants in the organism may be many times by magnitude higher than in the surrounding water, the likelihood of acute and chronic poisoning in organisms may grow (Daniel and Odioko, 2017). Additionally, many persistent organic pollutants are genotoxic, which means they have the capacity to alter the structure and integrity of DNA (González-Mille *et al*., 2010), and many genotoxic persistent organic pollutants have the capacity to increase the amount of DNA damage they inflict by producing more potent genotoxic intermediates that are produced metabolically throughout complex cellular processes (González-Mille *et al*., 2010; Berg *et al*., 2011; Baines *et al*., 2021). So, mutagenesis (changing genetic information), teratogenesis (producing developmental defects), clastogenesis (chromosome breaking), and carcinogenesis (generating cancer or tumors) may result from genotoxic persistent organic pollutants altering DNA (Vagi *et al*., 2021).

The degree of genetic material damage caused by numerous persistent organic pollutants, such as the relative increase in DNA adduct formation, DNA strand breaks, and/or micronucleus production, can be used to measure their genotoxicity (Göney and Gazeloğlu, 2020). Single-cell gel electrophoresis (or comet) assays, a method originally developed to identify DNA double-stranded breaks that happen in X-ray irradiated cells, are increasingly utilized to identify DNA damage in single cells. In the earliest stages of this experiment, single cells embedded in agarose were used to eliminate mechanical shear artifacts caused by

immersing the sample in a lysis solution containing high concentrations of salt and detergent. The cleaved DNA fragments are then violently forced away from the nucleoid cores by an electrophoretic field when the exposed DNA is exposed to it in a neutral environment. The assay is so named because, when stained with a fluorescent dye, the DNA resembles a comet when viewed under a fluorescence microscope (Nagarani *et al*., 2012; Møller *et al*., 2020). Additionally, the distance that the DNA has migrated from the core (i.e., the length of the comet tail) can be utilized to determine the severity of double-stranded DNA breaking because comet tail length is positively connected with the degree of DNA damage (Hussain *et al*., 2018). Additionally, it has been shown that the size and intensity of comet tail staining can be utilized to determine the degree of DNA damage (Pavlica *et al*., 2011). Although several adjustments to the comet test were subsequently proposed, the majority of current methods are virtually unchanged from those developed by Singh *et al*. (1988). Included in this is the procedure for the assay's alkaline unwinding, which permits sensitive detection of both single and double strand breaks as well as the generation of breaks at alkaline labile sites produced by DNA adduct creation.

The high-sensitivity detection of both double and single strand breaks is thus the key benefit of the comet test over other approaches. One break per chromosome or fewer than 200 breaks per cell have been reported as detection levels (Abdel-Gawad *et al*., 2011). Additionally, comet assay is frequently regarded as a quick, easy, reasonably priced, and trustworthy technique for identifying and repairing DNA damage within practically any population of eukaryotic cells. Furthermore, to detect these variations, only modest cell samples (< 10,000 cells) are needed, which is a crucial factor to take into account when the sample size is constrained (Çavas, 2011; Mota *et al*., 2019). It is possible to connect DNA strand breakage results with other cytogenetic reactions seen in single cells, such as the micronucleus induction assay, because the comet assay detects DNA damage at the single-cell level (Gomes, 2012).

The comet assay has been extensively used to evaluate the mechanisms of DNA damage and repair caused by radiation and chemicals, as well as to predict the genotoxic, mutagenic, and carcinogenic properties of a class of substances (De Lapuente, *et al*., 2015; Mota *et al*., 2019), though the primary focus of these studies has been on human and other mammalian cell types (Gajski, *et al*., 2019; Ayanda *et al*., 2021). The comet assay has been applied to aquatic vertebrates and invertebrates like fish, mussels, and oysters in the context of environmental toxicity (Çavas, 2011; Hussain *et al*., 2018; Møller *et al*.,2020). On the employment of the comet approach on aquatic organisms generally, there are, however, few research (Frenzilli and Lyons, 2013; Martins and Costa, 2017).

In contrast, the micronucleus assay can detect cellular genetic damage. The micronucleus assay is used when complete chromosomes or chromosomal fragments do not integrate into the daughter nucleus during prophase of cell division and remain in the cytoplasm throughout the cell life cycle.

For determining the genotoxic effects of environmental pollutants in contaminated effluents and water bodies, the micronucleus assay has proven to be the most effective (Çavas,2011; Hussain *et al*., 2018). It has been used in both laboratory and field investigations of amphibians, various fish species, and various bivalves (Bolognesi and Hayashi, 2011).

Fish, as aquatic creatures, are exposed to a wide spectrum of water pollutants and can thus serve as indicators for pollution assessment. Nile tilapia, *O. niloticus* (Linnaeus, 1758) (Perciformes, Cichlidae), is a domesticated fish that is commonly employed in ecological studies due to its ease of handling and maintenance in the laboratory. It easily adapts to confinement; it is susceptible to various contaminants; it has economic significance but is also quite invasive. In addition, Nile tilapia and its underlying tissues have been routinely utilized to assess the toxicity of various contaminants in aquatic habitats (El-Kasheif *et al*., 2018 Khalifa, 2017; Benli and Özkul, 2010).

To use DNA damage and induction micronucleus assays as biomonitoring tools, extensive research of the time-dependent effects of exposure to diverse genotoxic chemical concentrations are necessary. Furthermore, there has been little or no extensive examination of the association between degrees of DNA strand breakage and micronucleus induction. Bolognesi and Hayashi (2011) reported that the in micronucleus assay can be caused by chromosomal fragmentation. Chromosome fragmentation would most likely be preceded by DNA strand breaks, despite the fact that its mechanism has rarely been described. The aforementioned theory would be supported if there was a correlation between DNA stand breaks and micronucleus assay.

The current study aims to assess the dose-and time-dependent responses to DNA strand breakage and micronucleus formation in Nile tilapia blood cells, as well as the link between the frequency of micronucleus and the level of DNA strand breaks in fish blood cells. We subjected Nile tilapia to benzo(a)pyrene for 12 days and assessed the dose- and time-dependent responses of two cytogenetic endpoints in fish blood cells. The alkaline comet test was used to track DNA strand breaking, while the micronucleus assay was utilized to assess the degree of micronucleus formation.

MATERIALS AND METHODS

From November 2020 to December 2021, samples of Nile tilapia (*O. niloticus*) (Perciformes, Cichlidae) were taken from a generally clean area of the Shatt Al-Arab river. In the current trials, fish with average lengths and weights of 11.5-

10.5 cm and 115-270 g were utilized. Fish samples were collected from the water using the hand net. To get the fish to the lab, cool, sterile crates filled with river water were used.

For 10 days, live fish were acclimated to laboratory conditions in 20 L aquariums with a temperature of 25°C ±2 and a light-dark cycle of 12 hours of light and 12 hours of darkness. The water (Shatt Al-Arab river) of aquariums changed every morning. Electrical air pumps were used to provide oxygen to aquariums. Water had been adjusted for dissolved oxygen content (6 ±0.5 ppm) and pH (7.4-7.8). Fish were given commercial fish food twice a day, in the morning and the evening. Healthy fish were chosen for the trials after acclimatization based on their activity and skin texture.

In order to simulate chronic exposures that are likely to occur in the Shatt Al-Arab river and mimic a worst-case scenario that may occur in the future, the concentrations of 3, 15, and 30 µg/L of benzo(a)pyrene were chosen for the chronic toxicity test. These concentrations were based on the prevalence of benzo(a)pyrene in the surface water of Shatt Al-Arab river. To get nominal final concentrations (3, 15 and 30 µg/L) of benzo(a)pyrene, the appropriate quantities of benzo(a)pyrene dissolved in acetone (0.006% v/v) were added to the river water. An absolute control (just river water) and a solvent control were both used as controls. In glass aquariums measuring 40 cm 30 cm 30 cm, a total of 20 acclimated Nile tilapia fish were transplanted to each test concentration, along with the controls. Every day for 15 days, the test material was replaced using the semi-static (renewal) test. After each renewal, physical-chemical test media parameters were observed. Each day, fish were fed. Five fish were randomly selected from each replication after 15 days of treatment on days 0 (only untreated fish were sampled), 1, 3, 6, and 12 for micronuclei and comet assay. A one ml syringe was used to draw blood from the caudal veins of sedated fish, and the sample was then refrigerated for analysis at 4ºC in a refrigerator.

On the pre-cleaned slide, a very small smear of blood was created. The slide was allowed to dry naturally at ambient temperature. After 30 minutes of fixation in 70% ethanol, the slide was air dried for 24 hours before being stained with 10% Giemsa in phosphate buffered saline, pH 6.6, for 10 minutes. The slide was cleaned gently with water and air-dried. A total of 2000 erythrocytes from each slide were scored using a light microscope under oil immersion (1000X magnification). Micronucleus frequency was used to record the results. The frequency of micronucleus (%) was determined as follows: micronucleus (%) = (Number of cells containing micronucleus/Total number of cells counted) ×100 (Tates *et al*., 1980).

The alkaline comet test was carried out according to Singh *et al*. (1988). To avoid DNA damage from ultraviolet irradiation, all of the steps mentioned were carried out in low yellow light. Fish blood (10 µL) was diluted in phosphate buffered saline (pH 7.4). At 37ºC, 20 µL of this suspension was combined with 120 µL of 0.5% low melting point agarose dissolved in phosphate buffered saline. These suspensions were applied on slides that had previously been coated with 1.5% normal melting point agarose at 60ºC. The slides were then covered with cover slips and kept at 4ºC for 20 minutes to allow the agarose to solidify. The slides (after removing cover slips) were then placed in a lysing solution (220 mM NaCl, 9 mM EDTA, 0.9 mM Tris, 1% Triton X-100 and 10% DMSO, 0.9% sodium sarcosianate, pH 10.0) for an hour at 4°C while being shielded from light. After that, they were rinsed with distilled water to get the lysing solution off of them. Slides were put in the electrophoresis container and submerged for 20 minutes at 4 °C in the dark in a freshly made electrophoresis buffer solution (300 mM NaOH, 1 mM EDTA, 2% DMSO, pH> 13). At 4°C, electrophoresis was carried out for 20 minutes at a current of 25V (1.0 V/cm; 300 mA). The slides were then neutralized by submerging them for 15 minutes each in a neutral buffer (0.4 M Tris, pH 7.5) twice. The slides were then air-dried at ambient temperature for 10 minutes after being submerged in 100% ethanol at 4°C, and they were then refrigerated until cytological investigations. A fluorescence microscope with a green filter and an X40 magnification was used to examine slides stained with ethidium bromide (20 mg/ml). In 100 randomly selected, non-overlapping fish cells, DNA migration was observed. For each sample, one hundred cells were scored. The tail length (calculated from the middle of the head to the end of the tail), tail DNA content (tail % DNA), and Olive tail moment (tail length × tail DNA content) parameters evaluated.

The data from the assessment of the tail DNA content and length were used for the comet test without transformation, while the data from the assessment of the Olive tail Moment were log-transformed. The Kolmogorov-Smirnov test and the Barlett test were used, respectively, to confirm normality and the homogeneity of variance. The level of significance utilized in the two-way analysis of variance (ANOVA) to assess the independent effects of pollutant concentration and exposure on the degree of DNA strand breaking was 0.05. Pairwise Tukey tests were employed to find differences between particular treatment groups where significant effects were found. Anarcsine $\sqrt{P}$ angular transformation of micronucleus frequency was employed to stabilize the variance and approach normality because the sample size and micronucleus frequencies for the data obtained from the micronucleus formation test were low. Then, as mentioned before, two-way ANOVA and Tukey pairwise comparisons were used. By using Spearman rank correlation analysis, it was possible to discover the correlation between the comet characteristic known as the "Olive tail moment" and the micronucleus frequency.

RESULTS

Table (1), presents the micronucleus assay's outcomes, throughout the whole 12 days of exposure, both the untreated group and the solvent control group's micronucleus formation frequencies were low (range from 0.30 to 0.51%), and they were statistically similar (P> 0.05). The micronucleus frequencies of the benzo(a)pyrene-treated fish appeared to rise in a dose-dependent way at all times, resembling the outcomes seen in the comet experiment. Furthermore, independent of the length of exposure, analysis of the micronucleus frequencies between the various treatment groups revealed significant differences (P< 0.05) among the three benzo(a)pyrene-treated groups, as was found in the comet assay data. On days 3, 6, and 12, there were significant differences (P< 0.05) between the exposure groups to 0.3 and 30 µg/L of benzo(a)pyrene, according to comparisons of the micronucleus frequencies among the various treatment groups throughout certain time periods. Until the end of the experiment, micronucleus frequencies increased in all benzo(a)pyrene-treatment groups. Surprisingly, the highest micronucleus frequencies were seen on day 12, and data comparisons between time groups showed that regardless of the exposure concentration examined, the micronucleus formation frequencies on day 12 were significantly greater than those on days 0, 1 and 3.

Table (2), displays the results of the DNA strand breakage as mean tail DNA content, mean tail length, and mean Olive tail moment. No significant differences (P> 0.05) were discovered between the untreated and solvent control groups as all three assessed comet parameters remained constant and stable over the course of the 12-day exposure period in both groups. However, after exposure to benzo(a)pyrene, all of the measured comet parameters were shown to increase in a dose-dependent way at each time point, with the exception of the mean tail length at days 1 and 6. In addition, analyses of data from various treatment groups revealed that, independent of the length of exposure, there were significant differences (P< 0.05) between the 0.3, 3 and 30 µg/L benzo(a)pyrene-treated groups. Similarly, analyses of the treatment groups within specific time periods revealed significant differences (P< 0.05) on days 3 and 6 between the 0.3 and 30 µg/L benzo(a)pyrene-treated groups, as well as on day 12 across the three treatment groups. All measured comet parameters in the 0.3 µg/L group displayed an upward trend from days 0 to 3, but they were only significantly different from the control values (P< 0.05) on that day 3. By day 6, each parameter had basically reverted to background levels. From days 0 to 6, all comet parameters in the 3 µg/L exposure group were increased; however, from days 6 to 12, they were all lowered. On days 3, 6, and 12, all three parameters were significantly higher (P< 0.05) than the control levels. The mean tail DNA content and mean Olive tail moment at day 12 were, interestingly, both significantly lower (P< 0.05) than the corresponding values at day 6 when the comet parameters for days 6 and 12 were compared.

Table 1. The frequency of micronuclei in blood cells of Nile tilapia exposed to varying concentrations of benzo(a)pyrene for varying time periods in the control and treatment groups

Exposure time	Treatment	No. of fish	No. of cells observed	The Micronuclei frequency (%)
0 day	Control	5	2000	0.50^{c} ±0.78
1 day	Control	5	2000	0.42^{c} ±1.53
	Solvent control	5	2000	0.45^{c} ±2.82
	3 µg/l	5	2000	0.64^{c} ±1.17
	15 µg/l	5	2000	1.09^{ca} ±1.85
	30 µg/l	5	2000	1.41^{a} ±0.36
3 days	Control	5	2000	0.42^{c} ±0.23
	Solvent control	5	2000	0.41^{c} ±0.13
	3 µg/l	5	2000	1.55^{j} ±1.11
	15 µg/l	5	2000	4.62^{d} ±2.91
	30 µg/l	5	2000	7.67^{f} ±1.47
6 days	Control	5	2000	0.43^{c} ±1.83
	Solvent control	5	2000	0.46^{c} ±0.72
	3 µg/l	5	2000	2.97^{g} ±1.22
	15 µg/l	5	2000	5.98^{b} ±1.66
	30 µg/l	5	2000	11.02^{h} ±3.83
12 days	Control	5	2000	0.50^{c} ±3.19^{c}
	Solvent control	5	2000	0.44^{c} ±0.28
	3 µg/l	5	2000	6.65^{e} ±3.54
	15 µg/l	5	2000	10.02^{b} ±2.34
	30 µg/l	5	2000	13.84^{k} ±3.90

Lowercase letters indicate that there are statistically significant differences among different treatments at P < 0.05.

Finally, all comet parameters varied in the 30 µg/L exposure group in the same manner as they did in the 3 µg/L group. At all time points, the mean Olive tail moments and mean tail DNA content were both significantly higher than their control values (P< 0.05), while the mean tail lengths were significantly higher than the control values on days 3, 6, and 12. When the comet parameters at days 6 and 12 were compared, no significant differences were found.

Because significant positive relationships (high correlation) were found between the three parameters {tail length and tail DNA content (r= 0.737), tail length and Olive tail moment (r= 0.854), Olive tail moment and tail DNA content (r= 0.927) (P< 0.05)}, only the data for the Olive tail moment was used to determine the correlation between micronucleus frequencies and levels of DNA strand breakage. The Olive tail moment showed a significant and

positive connection between micronucleus frequencies and levels of DNA strand breakage (r= 0.763) (P< 0.05).

Table 2. Blood cell comets (tail DNA content, tail length, and olive tail moment) from Nile tilapia exposed to varying concentrations of benzo(a)pyrene for varying time periods in the control and treatment groups

Exposure time	Treatment	No. of fish	No. of cells observed	Tail DNA content (%)	Tail length (µm)	Olive tail moment (arbitrary unit)
0 day	Control	5	100	22.5^{j} ±3.2	11.6^{j} ± 2.9	530^{j} ± 4.0
1 day	Control	5	100	21.6^{j} ±1.4	11.1^{j} ±1.4	620^{j} ±1.0
	Solvent control	5	100	20.8^{j} ±2.3	12.0^{j} ±3.2	620^{j} ±3.0
	3 µg/l	5	100	25.6^{i} ±1.7	14.1^{i} ±3.7	830^{i} ±1.0
	15 µg/l	5	100	28.4^{i} ±2.9	13.6^{i} ±2.0	930^{i} ±3.0
	30 µg/l	5	100	35.3^{h} ±1.5	14.8^{h} ±3.2	1070^{h} ±3.0
3 days	Control	5	100	19.7^{j} ±1.7	10.2^{j} ±1.4	620^{j} ±2.0
	Solvent control	5	100	20.2^{j} ±1.9	11.7^{j} ±2.9	630^{j} ±2.0
	3 µg/l	5	100	33.6^{a} ±1.6	15.7^{a} ±1.0	1100^{a} ±1.0
	15 µg/l	5	100	39.2^{ab} ±0.8	16.6^{ab} ±1.6	1340^{ab} ±1.0
	30 µg/l	5	100	44.1^{b} ±1.4	20.2^{b} ±3.1	1660^{b} ±1.0
6 days	Control	5	100	21.0^{j} ±2.2	12.8^{j} ±1.8	650^{j} ±2.0
	Solvent control	5	100	19.9^{j} ±2.6	11.1^{j} ±1.4	620^{j} ±2.0
	3 µg/l	5	100	28.3^{c} ±0.7	13.1^{c} ±1.7	970^{c} ±1.0
	15 µg/l	5	100	46.3^{cd} ±0.9	19.1^{cd} ±1.3	1860^{cd} ±2.0
	30 µg/l	5	100	50.5^{d} ±1.5	19.2^{d} ±2.1	2100^{d} ±4.0
12 days	Control	5	100	22.8^{j} ±0.8	13.0^{j} ±4.7	610^{j} ±1.0
	Solvent control	5	100	20.2^{j} ±1.3	12.1^{j} ±1.9	600^{j} ±2.0
	3 µg/l	5	100	25.6^{e} ±1.1	12.5^{e} ±2.6	800^{e} ±2.0
	15 µg/l	5	100	33.8^{f} ±3.6	16.8^{f} ±1.2	1230^{f} ±4.0
	30 µg/l	5	100	44.7^{g} ±3.2	21.6^{g} ±3.5	1940^{g} ±5.0

Lowercase letters indicate that there are statistically significant differences among different treatments at P< 0.05.

DISCUSSION

Monitoring cytogenetic damage has frequently used fish blood cells. Blood cells are frequently used in this kind of research due to three favorable factors (Gomes, 2012; Hussain *et al*., 2018). Blood cells are a comparatively non-invasive source of material for bio-monitoring (Tan *et al*., 2013; Sayed, 2018) and they are also excellent for the comet and the micronucleus assays since they may be quickly and readily sampled without the need for cell dissociation (Hussain *et al*., 2018). These benefits have the advantage of speeding up slide preparation and streamlining sample processing. Furthermore, because cell dissociation is unnecessary, the amount of cellular damage generated by mechanical and/or proteolytic cell dissociation is reduced; low background levels of DNA damage, in particular, make it easier to recognize micronucleated cells. Finally, blood cells are thought to be more susceptible than other cells to external influences such genotoxic xenobiotics because of their multifunctional involvement in immunological defense, phagocytosis, transport, excretion, and detoxification of xenobiotics (Sayed, 2018).

In the control and the solvent control, the background amount of DNA damage was minimal. The lack of significant differences between the control and the solvent control suggests that the solvent had no impact on DNA strand breakage at the used concentration. It should be mentioned that the current study's DNA strand breakage levels were relatively high (the tail DNA content of the control group, for instance, was over 21%). Endogenous strand breaks or synthetic strand breaks produced during sample processing may be to blame for this. Using the comet assay, the control response of DNA damage in invertebrate cell types (such as liver cells and blood cells) is relatively high (Abdel-Gawad *et al*., 2011). According to Sahlmann *et al*. (2017), the substantially greater control response is a general feature of vertebrates and is most likely produced by the way their DNA is packed rather than the endogenous or manufactured strand breaks addressed previously. Other vertebrate research employing different DNA strand break detection tools have also demonstrated these phenomena of rather high control levels (Nagarani *et al*. 2012; Técher *et al*. 2017).

We have shown a dose-response association between the quantity of DNA strand breaks and ecologically relevant concentrations of benzo(a)pyrene using the comet assay. All comet parameters have demonstrated that, when evaluated without regard to the length of exposure, there are significant differences between the three benzo(a)pyrene-treatment groups (3, 15 and 30 µg/L). A clear dose-dependent response was also seen when the treatment groups were compared over specific time periods. This was shown by the significant differences on days 3 and 6 between the lowest (3 µg/L) and highest (30 µg/L) benzo(a)pyrene-treatment groups, as well as on day 12 between all three treatment groups. Additionally, a higher

dose of benzo(a)pyrene causes the animal's DNA damage to start sooner and to stay longer. A significant rise in the parameters of tail DNA content and Olive tail moment, which were measured on days 3 and only for the low dose, days 3, 6 and 12 for the medium dose, and at all time intervals for the maximum dose, can be seen as evidence for this. Our findings further imply that in Nile tilapia blood cells, strand-breaking effects are time-dependent. With their maximum values being observed on days 3 for the 3 µg/L group and 6 for the 15 and 30 µg /L groups, the tail DNA content and Olive tail moment values increased over time. For the 3 µg/L group, these recorded values gradually dropped to background levels, while for the 15 and 30 µg/L groups, they reached levels that were still significantly higher than background levels on day 12. The DNA repair theory put forth by Selby and Sancar (1994) and Fleck and Nielsen (2004), which suggests that a DNA repair system may be activated once the fish tissue has accumulated enough toxicant above a particular threshold level, can explain the time-dependent fluctuations in the levels of DNA strand breaks that have been observed (Singh *et al*., 1988). To facilitate DNA repair activity below this level, only a small quantity of DNA repair enzymes could be needed (Sancar and Sancar, 1988).

Unfortunately, the comet assay lacks uniformity despite its inherent advantages (Møller *et al*., 2020). Variations in the composition and pH of reagents (such as lysis solution and electrophoresis buffer) and the electrophoretic conditions (such as voltage, amperage, unwinding time, and running time) may affect the sensitivity of the assay by altering the shape and size of the resulting images. Many different protocols are currently being used in various laboratories (Christofoletti *et al*., 2009). Furthermore, the parameters employed to evaluate DNA migration have not yet been applied consistently (Kousar and Javed, 2015). For instance, several metrics, such as comet moment, tail length, tail DNA content (tail% DNA), and tail inertia, have been reported in a variety of methods (Tasneem and Yasmeen, 2018). Surprisingly, even when the same parameter is used, the definition can vary between laboratories. For instance, Schnurstein and Braunbeck (2001) defined tail length as the distance between the mean distribution of the head and the mean position of the tail, whereas Ashby *et al*. (1995) recommended that the tail length start at the trailing edge of the cell. Due to these variations, it is challenging to compare data from different laboratories employing different species and tissues when performing the comet assay.

The present work used simultaneous measurements of the tail DNA content, tail length, and Olive tail moment as endpoints of DNA damage, taking advantage of image analysis technologies that allow comets to be captured and stored for numerous investigations. This allowed at least intra-laboratory comparisons. According to our research, the Olive tail moment and the tail DNA content seem to produce identical outcomes and show similar tendencies. In fact, similar findings between these two characteristics have been reported in research using KB epithelial cells, hepatocytes, and gill cells from zebra fish (*Danio rerio*) (Schnurstein and Braunbeck, 2001). The measurements of the tail length, in contrast to the other two parameters, produced results that were distinct from those of the other two parameters. In particular, the tail length did not show a significant increase in the level of DNA strand breaks in the 30 g/L benzo(a)pyrene-treatment group on day 1 and also revealed inconsistent reductions in DNA damage on day 6 in the 15 µg/L and 30 µg/L treatment groups, in contrast to the tail DNA content and the Olive tail moment. Our investigation highlights the necessity for testing process standardization, as previously mentioned, by exposing the inconsistent nature of some comet detection characteristics within a particular data set (Devaux, 1998; Mitchelmore and Chipman, 1998). Because DNA migration is easily influenced by differences in the gel, the measurement of tail length has come under fire for failing to accurately reflect the extent of DNA damage. Earlier research has also suggested that tail DNA content and tail moment are the most accurate endpoints to express the observed DNA damage. Additionally, it has been discovered that comets with the same tail length but varied fluorescence intensity can frequently be seen (Schnurstein and Braunbeak, 2001).

Surprisingly, our findings contrast dramatically from those of a previous investigation, which found that the same nominal amounts of benzo(a)pyrene, exposure time, and regime induced a non-linear dose-dependent increase in the number of DNA strand breaks in milk fish liver cells (Palanikumar *et al*., 2012). In that investigation, it was found that levels of DNA strand breaks dramatically increased after one day of exposure to benzo(a)pyrene at 3 and 15 g/L. Furthermore, since no DNA strand breaks were noticed during the 12-day exposure period, Ching *et al*. (2001) showed that the DNA repair machinery in the liver cells of the 30 g/L exposure group was immediately activated. The discrepancies between the current and earlier findings imply that tissue-specific responses to DNA strand breaks occur. In fact, Monteith and Vanstone (1995) showed that distinct DNA strand breakage reactions can occur depending on the cell type. Similar to this, Mitchelmore and Chipman (1998) have previously shown that in vitro exposure to benzo(a)pyrene (0-200 µM) causes significant dose-dependent increases in DNA strand breaks in the digestive gland cells of the brown trout (*Salmo trutta*), whereas the hemocytes of this organism only exhibit a slight and statistically insignificant response. These results indirectly support the notion of tissue-specific differences in fish by demonstrating that digestive gland cells had higher metabolic activity for benzo(a)pyrene than hemocytes.

The rapid rise in DNA damage (and associated chromosomal damage) after mussels were exposed to benzo(a)pyrene has previously been theorized to be caused by metabolically activated intermediates like quinone and

reactive byproducts, such reactive oxygen species (ROS) (Venier *et al*., 1997). Since circulating cells are thought to metabolize xenobiotics, benzo(a)pyrene metabolism has already been shown to occur in the digestive gland of mussels (Martinez and Livingstone, 1995), and the same metabolic mechanisms are thought to operate in the hemocytes (Kennedy et al., 1991). Vertebrates and invertebrates may, however, have quite different xenobiotic metabolisms in circulating cells. For instance, benzo(a)pyrene (0-200 µM) only slightly and statistically insignificantly increased the proportion of DNA strand breakage in blood cells from brown trout (*S. trutta*), indicating that circulating cells had poor metabolic activity for genotoxicants (Mitchelmore, and Chipman, 1998). Furthermore, the same study found that a single intraperitoneal injection of 50 mg/kg of benzo(a)pyrene for 24 hours can result in significant increases in the proportion of DNA strand breaks in brown trout blood cells, and it was hypothesized that exposure of the circulating cells to reactive metabolites produced by hepatic metabolism was the primary cause of cellular DNA damage in circulating cells. After being exposed to benzo(a)pyrene in vivo, significant DNA damage has been reported in the hemocytes of the oyster (*Crassostrea virginica*), but not in the blood cells of the flounder (*Pseudopleuronectes americanus*). This suggests that the circulating cells of an invertebrate may be more sensitive to DNA damage than the cell of a vertebrate (Abdel-Gawad *et al*., 2011). More research is needed to identify the proportional benzo(a)pyrene-metabolizing potential of circulating cells in invertebrates and vertebrates. Regardless of the metabolic mechanism for such differences between tissues, the current study's dose- and time-dependent DNA strand breakage implies that the response in Nile tilapia blood cells will provide a superior genotoxicant monitoring tool than liver cells.

The micronucleus assay was used in the second phase of the current investigation to evaluate benzo(a)pyrene's clastogenic qualities. The data provided here should be accurate because a sample size of 2000 cells from each of 4 individuals was used, which is higher than the minimum recommended sample size of 500 cells from each of 4 individuals by Mersch and Beauvais (1997). Along with the previously mentioned benefits of using blood cells to evaluate genotoxicity, it has also been proposed that, due to blood cells' non-granular nature and relatively low levels of spontaneous micronucleus, they can be used to identify micronuclei more accurately than the more popular gill cells (Ergene *et al*., 2007; Hayashi, 2016). In fact, the spontaneous micronucleus frequencies in the current study varied from 0.42 to 0.50%, and these numbers are significantly lower than those of the gill cells of Nile tilapia (Ergene *et al*., 2007). In additional researches using various fish species, comparable tissue-specific changes in the rate of spontaneous micronucleus formation in gill cells and blood cells were also discovered (Çavas *et al*., 2005; Mert *et al*., 2014). It's interesting to note that the frequencies of spontaneous micronucleus formation in the blood cells documented here are likewise lower than those values found in other research utilizing blood cells from different fish species. The current study's decreased spontaneous frequency of micronucleus formation is probably the result of either Nile tilapia's naturally lower spontaneous rates of formation, the assay's enhanced experimental protocols, or both.

According to the results of the current investigation, benzo(a)pyrene exposure led to a considerable induction of micronuclei in the blood cells of Nile tilapia. It has already been proposed that DNA double strand breaks are the primary cause of the induction of micronuclei (da Silva Souza and Fontanetti, 2006).

Although the mitotic rate of blood cells in Nile tilapia fish has rarely been investigated, the cells should divide rapidly in response to chemical stress, and Özkan *et al*. (2011) has shown that blood cells are rapidly recruited during defensive and immunological reactions. According to the current study, blood cells should divide within 72 hours because the first significant micronucleus responses were seen on the third day after exposure. In fact, according to some research, the mitotic rate of blood cells could be as quick as 48 hours (Hussain *et al*.,2018). Additionally, significant micronucleus induction in blood cells of several fish subjected to benzo(a)pyrene and other aromatics for 48 hours was discovered (Palanikumar *et al*., 2012; Bücker *et al*., 2012). However, it doesn't seem that this quick mitotic activity causes the blood cell to respond with a lot of micronuclei. In the present investigation, only 13.84% mean micronucleus frequencies were found to be the highest. The poor micronucleus induction capacity appears to be a characteristic of all Nile tilapia cell types, as similarly low values have previously been seen in the gill cells of Nile tilapia treated to a genotoxicant mixture (Çavas *et al*., 2005; Elsappah, 2013).

The Nile tilapia blood cells showed clear dose-and time-dependent responses to genotoxicant (benzo(a)pyrene) exposure in the results of the comet assay-based and micronucleus frequency-based study, which are presented here. These responses in this cell type can be sensitive indicators (biomarkers) of a genotoxicant within an environmentally realistic range. The comet assay parameter "Olive tail moment" and the frequency of micronucleus formation are significantly correlated, and the assays offer a set of practical, very sensitive, non-invasive monitoring instruments of environmental exposure to genotoxicants.

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