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Induction of micronuclei and other nuclear abnormalities in peripheral erythrocytes of Nile tilapia, *Oreochromis niloticus*, following exposure to sublethal cadmium doses

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Abstract: The genotoxic effects of sublethal doses of cadmium in peripheral erythrocytes of *Oreochromis niloticus* was investigated using micronucleus (MN) and nuclear abnormalities (NAs) tests. For this reason, the fish were exposed to medium changes of 0.5 and 1.0 mg/L doses of cadmium (Cd) during an average treatment period of 10 days. On the 2nd, 4th, 6th, and 10th days of the test period, the erythrocytes, including the micronuclei (MN) and other nuclear abnormalities such as lobbed nuclei (LB), blebbed nuclei (BL), and notched nuclei (NT), were counted and pointed out. As a positive control, a 4 mg/L dose of cyclophosphamide was used. Exposure to doses of cadmium caused significantly increased ($P < 0.05$) micronuclei in peripheral erythrocytes of fish. It was also observed that the application of a 1.0 mg/L dose of Cd produced more of an effect on MN frequencies than the application of a 0.5 mg/L dose. The maximum frequencies of MN were recorded on the fourth day of the experiment period. Exposure to doses of cadmium caused significantly increased ($P < 0.05$) NA (LB, BL, and NT) frequencies and, as with the MN results, the 1.0 mg/L dose of Cd had more of an effect than the 0.5 mg/L dose on NA frequencies. The maximum frequencies of NAs were recorded on the fourth day of the experiment. As a result of these doses of Cd, the MN and NA frequencies in peripheral erythrocytes of fish were observed to increase in relation to both the time and dose applied. However, a gradual trend of decrease was also observed starting from the sixth day.

Key words: Micronucleus, nuclear abnormalities, cadmium, *Oreochromis niloticus*, erythrocyte

Nil tilapya (*Oreochromis niloticus*)'sı periferal eritrositlerinde mikronukleus ve diğer nukleus anomalilerinin oluşumu üzerine subletal kadmiyum derişimlerinin etkileri

Özet: Kadmiyumun subletal derişimlerinin *Oreochromis niloticus*'un peripheral eritrositlerin üzerine genotoksik etkileri, mikronukleus (MN) ve nukleus anomalileri (NAs) testleri yapılarak araştırılmıştır. Bu amaçla, balıklar 10 gün süreyle 0,5 ve 1,0 mg/L kadmiyum (Cd) ortam derişimlerine maruz bırakılmıştır. Deneyin 2. 4. 6. ve 10. günlerinde mikronukleus ve diğer nukleus anomalileri olan loblu nukleus (LB), biloblu nukleus (BL) ve çentikli nukleus (NT) içeren eritrositler sayılarak tesbit edilmiştir. Pozitif kontrol amacıyla 4 mg/L cyclophosphamide içeren ortam derişimi kullanılmıştır. Uygulanan Cd derişimleri peripheral eritrositlerde MN frekansını önemli oranda ($P < 0,05$) attırmış ve 1,0 mg/L Cd derişimi, MN frekansı üzerinde 0,5 mg/L Cd derişimine göre daha etkili ($P < 0,05$) olmuştur. Deney süresince, en yüksek MN frekansı 4. günde saptanmıştır. Cd derişimlerinin her ikiside nukleus anomalileri (LB, BL, NT)

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frekansını önemli oranda arttırmış (P < 0,05) ve 1,0 mg/L Cd derişimi NAs frekansları üzerinde 0,5 mg/L Cd derişimine göre daha etkili (P < 0,05) olmuştur. En yüksek nukleus anomalileri frekansı deneyin 4. gününde belirlenmiştir. Sonuç olarak, Cd derişimleri etkisiyle, peripheral eritrositlerde MN fekansları ve NAs frekansları derişime ve zamana bağlı olarak yükselmiş, fakat 6. günden itibaren azalma eğilimi göstermiştir.

Anahtar sözcükler: Mikronukleus, nukleus anomalileri, kadmiyum, *Oreochromis niloticus*, eritrosit

Introduction

Various industrial and agricultural activities increase pollution, particularly in the aquatic environment, which is contaminated by various toxic chemicals from the discharge of wastewaters and agricultural drainage (Isani et al., 2009). Heavy metals are common aquatic pollutants that enter aquatic systems via natural and anthropogenic sources. Chronic exposure to and accumulation of heavy metals by aquatic biota can result in tissue burdens that produce adverse effects not only in the exposed organisms, but also in other organisms such as human beings (IARC, 1993). Different compounds in polluted water are reported to damage the genetic material of exposed organisms and therefore cause genotoxic effects. Genotoxic studies on aquatic organisms exposed to polluted waters containing heavy metals have shown indications of DNA strand breakages and fish are employed as sensitive indicators for their genotoxic and mutagenic effects (Pruski and Dixon, 2002; Yadav and Trivedi, 2009).

Cadmium is a noted environmental toxicant among heavy metals. Its ability to accumulate in living organisms is a significant threat to the environment and public health (Kasuba and Rozgaj, 2002). Cadmium is a non-essential metal, widely distributed in the aquatic environment as a result of natural and anthropogenic activities (Isani et al., 2009). It has a high carcinogenic potential for humans and laboratory animals (Waisberg et al., 2003).

The micronucleus (MN) test, one of the most popular tests of environmental genotoxicity, has served as an index of cytogenetic damage (Fenech et al., 2003; Udroiu, 2006; Iarmarcovai et al., 2008). Micronuclei are cytoplasmic chromatin masses with the appearance of small nuclei that arise from chromosome fragments or from intact whole chromosomes lagging behind in the anaphase stage of cell division. Their presence in cells is a reflection of structural and/or numerical chromosomal aberrations arising during mitosis (Heddle et al., 1991; Bolognesi et al., 2006). The formation of morphological nuclear abnormalities (NAs) was first described in fish erythrocytes by Carrasco et al. (1990). NAs, including lobbed (LB), blebbed (BL), and notched (NT) nuclei and binucleated (BN) cells, have been used by several authors as possible indicators of genotoxicity (Da Silva Souz and Fontanetti, 2006). Several studies have shown that erythrocytes of fish present a high frequency of micronuclei and nucleus abnormalities after exposure to different heavy metals under both field and laboratory conditions (Al-Sabti and Metcalfe, 1995; Çavaş et al., 2005; Isani et al., 2009; Yadav and Trivedi, 2009). For the determination of genotoxic effect in fish, the micronucleus test as well as the study of the abnormal shape of nuclei is a suitable measure with which the presence or absence of genotoxins can be detected in water. The detection of MN and NAs in fish help us to assess the status of water quality as well as the health of a particular species and any potential risk it might have after consumption (Talapatra and Banerjee, 2007).

Nile tilapia (*Oreochromis niloticus*) is a good model for toxicological studies due to its easy reproduction and higher adaptation (Wu et al., 1999). In this study, fish were exposed to in vivo sublethal doses of cadmium (0.5 and 1.0 mg/L) for 2, 4, 6, and 10 days. Genotoxic effects were investigated in peripheral erythrocytes by using MN and NA tests; MN and NA frequencies were then evaluated in comparison with positive and negative control groups.

Materials and methods

The fish (*O. niloticus*) were obtained from Mersin University's fisheries faculty and aquaculture department. The fish had an average weight of 30.06 ± 1.40 g and an average length of 12.56 ± 0.80 cm. Fish were fed with commercial fish food at 2% body weight per day (Pinar, Turkey), and acclimatized under laboratory conditions for 2 weeks in a large tank containing dechlorinated tap water (pH: 7.85; alkalinity: 154 mg/L $CaCO_3$; DO: 6.70 mg/L; temperature: 22 °C) before the experiment. The photoperiod used provided 12/12 h dark/light. The aquaria used in our study were made of glass, offered constant aeration, had physical dimensions of 100 × 40 × 40 cm, and a 120 L capacity.

The fish were divided into 4 groups each group containing 12 individual fish. Group I: Negative control (only tap water was used); Group II: Positive control (4.0 mg/L cyclophosphamide; Sigma); Group III: 0.5 mg/L $CdCl_2$ (Sigma); and Group IV: 1.0 mg/L $CdCl_2$. The treatment concentrations of cadmium were selected based on data from the literature (Almeida et al., 2001). The water in each aquarium was replenished every 2 days in order to keep the metal concentrations constant. During the experiment, no mortality was observed.

Peripheral blood samples were obtained from the caudal vein of fish on the 2nd, 4th, 6th, and 10th days of the exposure period. For each group and at every interval, 3 fish were used and the blood was immediately processed for MN and NA tests. At each assessment, 5000 cells/fish were analyzed, offering 15,000 erythrocytes for each group. The blood samples were dropped on 5 clean microscopic slides for each fish, fixed in pure ethanol for 10 min and left to air-dry at room temperature. Afterwards, this was followed by 10% Giemsa (w/v) staining for 10 min. In each preparation, 1000 erythrocytes were examined under the light microscope using 1000× magnification to determine the frequencies of micronucleus and nuclear abnormalities. For the scoring of micronuclei, the following criteria were adopted from Al-Sabti and Metcalfe (1995): MN must be smaller than one-third of the main nuclei and clearly separated from the main nuclei. NAs were classified according to Carasso et al. (1990) (Figure 1A-D).

The one-way analysis of variance (ANOVA), Student-Newman-Keuls (SNK) test was used to compare the mean differences in MN and NA frequency between groups and exposure periods. Results of $P < 0.05$ were considered statistically significant. The frequencies of MN and NAs are reported as mean ± standard error for each experimental group.

Results

The result of micronucleus and nuclear abnormalities (LB, BL, and NT) in peripheral erythrocytes of *O. niloticus* exposed to 0.5 and 1.0 mg/L of cadmium concentrations for 2, 4, 6, and 10 days were shown in Figure 2A-D, respectively. In both of the groups receiving Cd doses as well as in the positive control group, frequencies of micronuclei were observed to be significantly higher ($P < 0.05$) when compared to the negative control group (Figure 2A). The cadmium dose of 0.5 mg/L significantly increased ($P < 0.05$) the micronuclei frequencies in erythrocytes on the 2nd, 4th, and 6th days, but there was no significant difference on the 10th day in comparison with the negative control group. Subjects receiving the 1.0 mg/L dose of Cd showed more of an increase than those on the 0.5 mg/L dose in all of the exposure periods. Especially on the 4th and 6th days, the results were similar to those of the positive control group (Figure 2A). The maximum frequency of MN was recorded on the 4th day of exposure to concentrations of cadmium. MN frequencies in erythrocytes increased depending on dose and time but decreased starting from 6th day in all of the treatment groups.

In both of the groups receiving cadmium doses and also in the positive control significantly higher ($P < 0.05$) frequencies of NAs were observed when compared to the negative control (Figure 2B-D). The frequencies of lobbed nuclei, blebbed nuclei, and notched nuclei were significantly higher ($P < 0.05$) in all experimental groups when compared to the negative control group. Nuclear abnormalities frequencies were found to be significantly increased ($P < 0.05$) on the 4th and 6th days compared to other exposure days. In general, similar to the results obtained for MN, NA frequencies in erythrocytes increased dose- and time-dependently but decreased starting from the 6th day in all treatment groups.

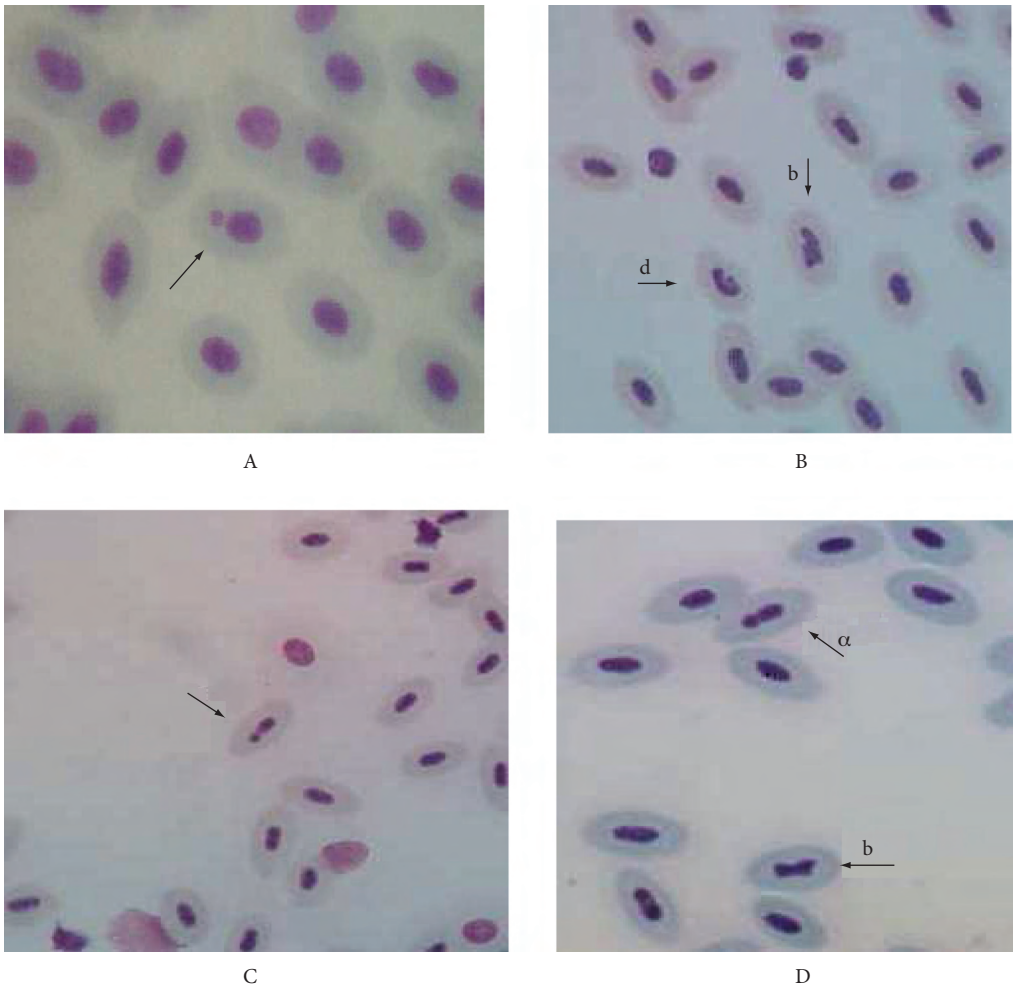

Figure 1. Micronuclei and nuclear abnormalities in peripheral blood erythrocytes of *O. niloticus* exposed to cadmium concentrations. **A:** Micronucleated erythrocyte, **B:** a) Notched nuclei b) Lobbed nuclei **C:** Blebbed nuclei **D:** a) Blebbed nuclei b) Lobed nuclei.

Discussion

The results of our study showed the induction of micronuclei in peripheral erythrocytes of *O. niloticus* when exposed to different concentrations of cadmium. In line with our findings, several authors have previously been able to demonstrate that the micronuclei test in fish erythrocytes and other tissue cells yielded positive results after the administration of cadmium. Cadmium induced significant micronuclei formation in different tissues of *Oreochromis mossambicus* (Chandra and Khuda-Bukhsh, 2004), *Salmo trutta,* and *Phoxinus phoxinus* (Sanchez-Galan et al., 1999). Similarly, Çavaş et al. (2005) reported an increase in the induction of MN in peripheral blood erythrocytes, gill epithelial cells, and liver cells of *Cyprinus carpio*, *Carassius gibelio*, and *Corydoras paleatus* with exposure to doses of Cd. They suggested that the mechanism of cadmium genotoxicity is mainly conditioned by single strand breaks in DNA through the direct cadmium-DNA interactions as well as by the action of incision nucleases and/or DNA-glycosylase during DNA repair (Privezentsev et al., 1996; Lutzen et al., 2004).

It has been reported that most of the toxic chemicals that produce genotoxic effects have been known to form reactive oxygen species (ROS) as well as electrophilic free-radical metabolites that interact with DNA to cause disruptive changes (Chandra

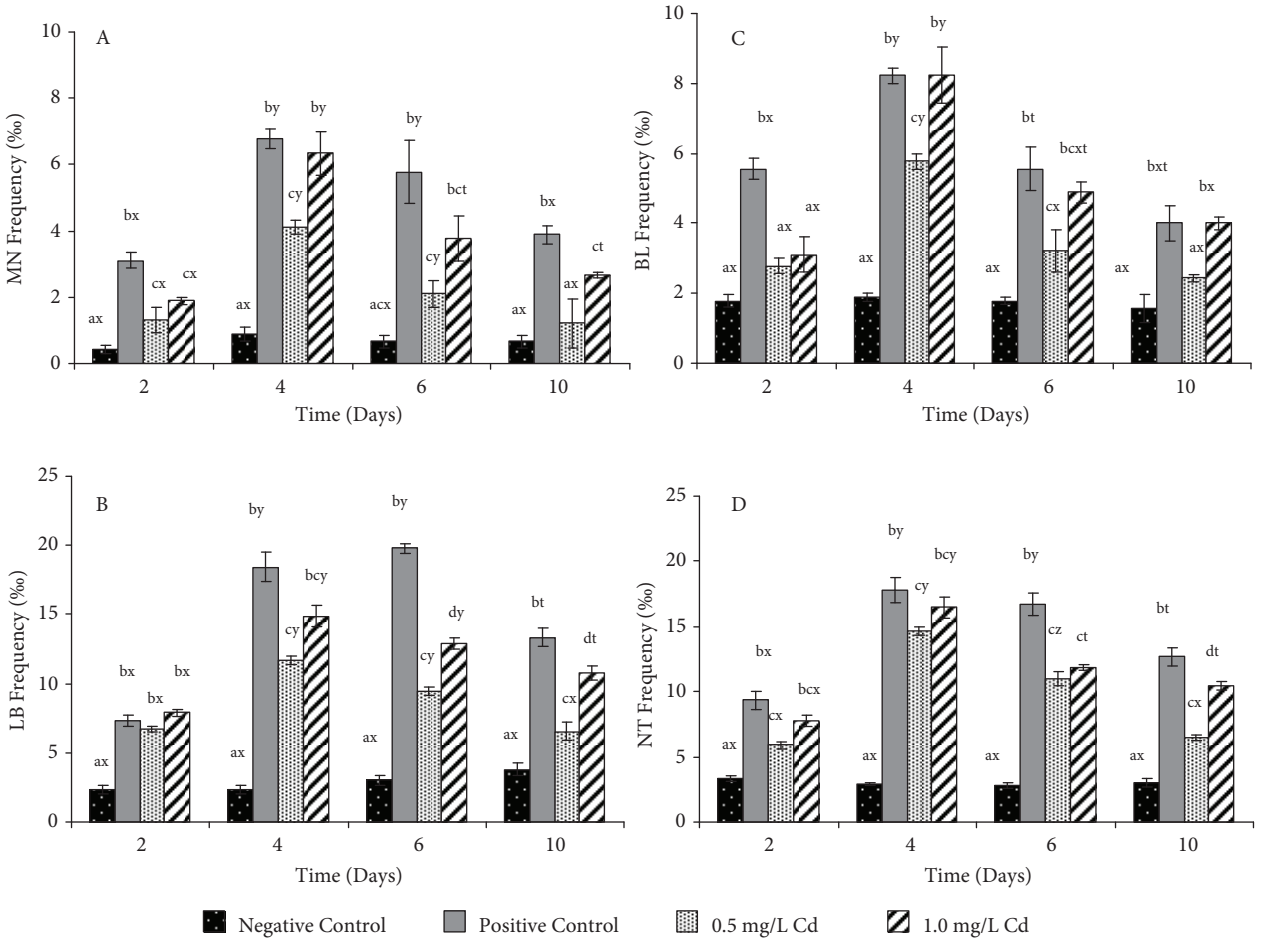

Figure 2. Frequencies of MN and NAs in peripheral blood erythrocytes of *O. niloticus* exposed to cadmium concentrations. **A**: Micronuclei frequencies; **B**: Lobbed nuclei frequencies; **C**: Blobbed nuclei frequencies; **D**: Notched nuclei. Mean ± SE a, b, c, d: indicate a significant difference ($P < 0.05$) between means recorded for the same day in each group.

x, y, z, t: indicate a significant difference ($P < 0.05$) between means recorded for the different days in same group.

and Khuda-Bukhsh, 2004). Cadmium is able to induce the production of a variety of ROS including H_2O_2, O_2^-, and OH^- (Bagchi et al., 1997; Waisberg et al., 2003). This provides the principal explanation for the cellular toxicity of this heavy metal (Bertin and Averbeck, 2006). Although the mechanisms leading to the increase of oxidant stress remain largely unknown, ROS production is observed after exposure to Cd and leads to a reduction in cellular antioxidants and lowers cellular defense against oxidative stress (Bertin and Averbeck, 2006; Liu et al., 2009). It was indicated that the MN induction may be also interpreted as a consequence of oxidative stress caused by Cd (Çelik et al., 2009).

Our results also showed that the exposure of fish to concentrations of Cd induced elevated levels of other nuclear abnormalities. Several authors have reported that chemicals induce nuclear abnormalities in different tissues of fish (Palheres and Grisolia, 2002; Çavaş et al., 2005; Talapatra Banerjee, 2007). The present study is in agreement with previous studies that indicate that metal genotoxins are mainly responsible for these nuclear alterations. Arkhipchuk and Garanko (2005) also showed a significant increase in double nuclei frequencies in blood and different tissue cells of fish treated with sublethal concentrations of Cd. According to Shimizu et al. (1998), the formation of these abnormalities would

represent a way to eliminate any amplified genetic material from the cell nucleus. A large number of chemicals may interfere in the DNA synthesis of an exposed organism, which could result in nuclear abnormalities (Da Silva Souz and Fontanetti, 2006; Ventura et al., 2008). It was reported that nuclear alterations such as notched or binucleated cells, which may be associated with aneuploidy, might have originated as a result of a failure of tubiline (Ventura et al., 2008) and the abnormal cells originate due to the difficulty of forming mitotic fuses caused by aneugenic actions of chemicals (Fernandes et al., 2007). Although the mechanisms responsible for NAs have not been fully explained, these abnormalities are considered to be indicators of genotoxic damage and, therefore, they may complement the scoring of micronuclei in routine genotoxicity surveys (Bolognesi et al., 2006; Çavaş, 2008; Strunjak-Perovic et al., 2009).

This study revealed that the maximum frequencies of MN and NAs were recorded on the fourth day of exposure to concentrations of cadmium. MN frequencies in erythrocytes increased according to both dose and time but gradually decreased starting from the sixth day of the exposure period. Das and Nanda (1986) reported a dose-dependent and time-dependent increase in the induction of micronuclei in peripheral blood of *Heteropneustes fossilis*. Yadav and Trivedi (2009) found that frequencies of micronuclei were increased in *Channa punctata* after 96 h of exposure to heavy metal, but that the frequencies decreased gradually with continued exposure. Studies on the rate of MN induction in various fish species have generally shown a peak between the first and fifth days of the treatment (Al-Sabti and Metcalfe, 1995; Palhares and Grisolia, 2002). It appears that a peak in micronucleated erythrocytes occurs 1 to 5 days after exposure, but in most species it takes place after 2 or 3 days and the time required to reach the peak of micronucleus induction in peripheral blood varies greatly among teleosts (Udroinu, 2006). In accordance with the results of our study, a gradual decrease was also observed after increased frequencies of MN and NAs (Çavaş et al., 2005; Yadav and Trivedi, 2009). Nepomuceno et al. (1997) have suggested that the higher pollutant concentration might inhibit normal cell division, damage erythrocyte chromosomes, and interrupt DNA duplication, causing micronuclei frequencies to more or less decline. Then micronuclei frequencies tend to even out and fish might promote some defensive mechanism to reduce some of the metal residues in the body in order to stabilize the micronuclei frequencies (Nepomuceno et al., 1997).

In our study, the positive control test group receiving cyclophosphamide showed a significant potential genotoxicity to *O. niloticus* when compared with the results from the negative control group. Negative and positive controls have been used in all mutagenic tests (Çavaş et al., 2005). Cyclophosphamide is an alkylating agent and, thus, it is a clastogenic agent for various animal species and is usually used as a positive control in vivo tests of short duration. It causes alkylation of the purine ring and, as a result, there is a miscoding and blockade of DNA replication (Çavaş et al., 2005).

In conclusion, the results of the present study demonstrated the induction of genotoxic damage as revealed by the micronucleus and nucleus abnormalities assays on fish under exposure to cadmium. The use of fish as biomarkers of the effects of pollution is of increasing importance and can permit the early detection of aquatic environment pollution. These organisms respond to toxic agents in a way that is similar to that of higher vertebrates, which can allow for the assessment of substances that are potentially hazardous to humans. Therefore, we need to determine the genotoxic effects of chemicals such as heavy metals.

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