Increase of altered nuclei in peripheral erythrocytes of *Oreochromis niloticus* following exposure to sub lethal concentrations of crude oil

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1. Introduction

Crude oil includes a variety of components, such as poly aromatic hydrocarbons (PAHs), nitrogen–oxygen mixtures and heavy metals which are the major elements of petroleum hydrocarbon pollution in aquatic ecosystems. Different PAHs can have dangerous consequences due to oxidative biotransformation, which produces highly DNA-reactive metabolites that are known carcinogenic and mutagenic chemicals (Djomo et al., 2004; Oliva et al., 2012). Genotoxic potency of these metabolites is expressed in fish due the relatively low metabolic rate of biotransformation of PAHs and has been confirmed by different studies (Baršiene et al., 2006; Çavaş & Ergene-Gözükara, 2005; Kanthi et al., 2015; Kanthi & Jayaweera, 2021). Micronuclei assays and other erythrocyte nuclear abnormality (ENA) assays are most common and promising biomarkers for assessing genotoxicity in fishes (Baršiene et al., 2004). In recent years, several investigations have shown the existence of nuclear abnormalities (NAs) other than micronuclei in cells of fish exposed to genotoxic chemicals could be used as effective tool for assess genotoxicity (Ayllon & Garcia-Vazquez, 2000; Baršienė & Andreikėnaitė, 2007; Çavaş & Ergene-Gözükara, 2003; 2005). Among those erythrocyte abnormality types, altered nuclei (AN) have been identified as a type of ENA which consisted with several nuclear lesions which are readily available in exposed fish peripheral blood erythrocytes (Kanthi et al., 2015). Furthermore, altered nuclei are a combination of blebbed nuclei, notched nuclei, lobed nuclei, and kidney shaped nuclei (Çavaş & Ergene-Gözükara, 2003). Analysis of AN is used for in situ genotoxicity assessment in aquatic media (Hose et al., 1987; Al- Sabti & Hardig, 1995; Al-Sabti & Metcalfe, 1990). Since fish often respond to toxicants in a manner like higher vertebrates, they can be used to screen for chemicals that have the potential to cause teratogenic and carcinogenic effects in humans. The aim of this study was to investigate the frequency trends of altered nuclei over the exposure period in the fish species *Oreochromis niloticus* treated with sub lethal concentrations of crude oil under controlled conditions.

2. Materials and Methods

Forty-day laboratory experiment was carried out using advanced fingerlings of (*Oreochromis niloticus*) (n=9 per tank; two replicates) which were exposed to sub lethal crude oil concentrations (T1 = 3ppm and T2 = 15ppm, v/v% in freshwater) and the effects were compared with a control group (C) not exposed to crude oil. Water and crude oil exposure renewal was done after each 5 days of exposure. Water quality (Temperature, pH, Salinity, Total ammonia) was monitored regularly throughout the experimental period. Feeding was done as once per day and daily fecal matter removal procedure was carried out.

Peripheral blood sampling (n = 10 per group) was carried out on the 5^{th}, 10^{th}, 20^{th} and 40^{th} day of the exposure period to prepare blood smears. For the blood collection, randomly selected fish were anaesthetized by using benzocaine solution. Blood samples were obtained by caudal vein puncture (2 slides per fish), directly smeared on the slide, and air-dried overnight. The air-dried blood smears were fixed using absolute methyl alcohol (Methanol) before the staining of the smears. Then slides were stained with 10% Giemsa solution for 60 minutes. Then slides were rinsed with distilled water and air-dried at room temperature overnight. Counting of

altered nuclei was done on each slide, areas with a uniform spread in monolayer without overlapping cells according to the classification key introduced by [Figure 1] (Çavaş & Ergene-Gözükara, 2003).

Different altered nucleus types were enumerated while counting up to a total of 5000 RBCs (per fish) and Frequency of total AN (per 1000 RBCs per fish) was calculated. The trends of frequency of AN were statistically analyzed by using Kruskal-wallis test and Mann-whitney U test for pairwise comparisons between sampling dates (SPSS ver.16) and represented with graphical illustrations.

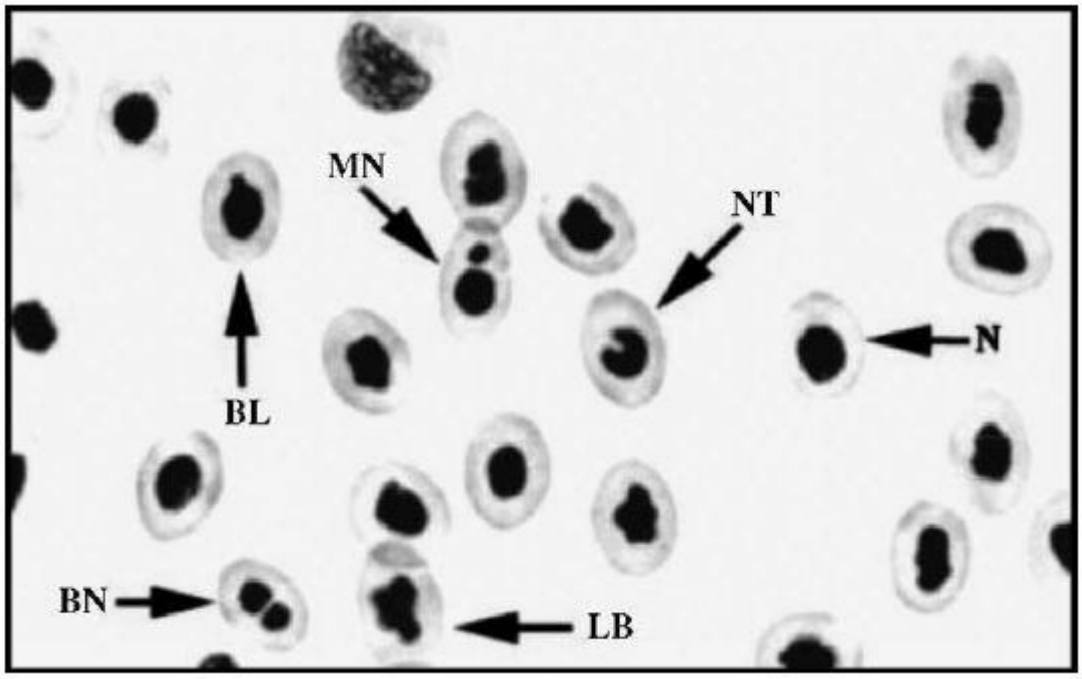

Figure 1. The micrograph adapted from Cavas et al., (2003) to be used as the guidelines for identify different altered nuclei, N: Normal red blood cell, BL: blebbed nuclei; LB: lobed nuclei; NT: notched nuclei/ kidney shaped nuclei in peripheral blood erythrocytes of *O. niloticus*

Table 01. Summary description statistics of altered nuclei in peripheral blood of O. *niloticus* among four sampling times over 40-day exposure period in experimental groups (Mean ± SD calculated from 10 fish per group). Results from Mann- Whitney U test for comparison between pairwise are indicated by the superscript letter (a, b, c, d), where shared letter within rows indicate homogeneity

Sampling date / Exp. group	5th day	10th day	20th day	40th day	P value
Control	0.52± 0.329^{a}	2.10±2.104^{b}	0.56± 0.350^{a}	0.44± 0.263^{a}	0.018
Treatment 1 (3ppm)	2.26±1.181^{ab}	2.09± 1.908^{ab}	1.60±0.805^{abc}	1.02±0.537^{d}	0.054
Treatment 2 (15ppm)	3.40±2.268^{a}	5.18± 2.292^{ab}	2.74±1.095^{acd}	2.56±1.061^{acd}	0.034
P value	< 0.001	< 0.001	< 0.001	< 0.001	

3. Results and Discussion

There was no significant difference ($P > 0.05$) within each experimental group among different sampling points for selected water quality parameters. Therefore, the quality of water in control and treatment tanks did not vary significantly during the experimental period, except for the suspensions of oil droplets in treatment groups because of the water-soluble fraction of PAHs in crude oil.

Normal red blood cells were observed to have a spherical, clearly demarcated nuclear surrounded by cytoplasm (Figure 1) and normal RBCs were highly prevalent in the control group. Blebbed nuclei, notched nuclei, lobed nuclei, and kidney shaped nuclei were observed as altered nuclei on each sampling date in all experimental groups. Kidney shaped altered nuclei type was highly available in treated groups than in control group. In each sampling date there was a significant difference in altered nuclei among the experimental groups ($P<0.001$) [Table 1]. According to the pairwise comparison (control vs. treatment 1control vs. treatment 2/ treatment 1 vs. treatment 2) on each sampling date between control and treated groups, significant mean frequencies of AN were perceived in each comparison except between control and treatment 1 on the 10^{th} sampling date [Table 1]. The mean frequency of AN was high in the treatment 2 (15ppm) group compared to the treatment 1 (3ppm) group throughout the exposure period [Figure 2].

According to the statistical results for comparison of mean frequencies of AN among sampling dates of each group, control and treatment 2 groups showed significant ($P < 0.05$) variation of altered nuclei counts with respect to exposure period [Table 1]. In the control group, though the frequencies were low in counts compared to treated groups, a substantial number of altered nuclei were observed on 10^{th} sampling date. Moreover, after the 5^{th} day of the experiment, there was considerable upwelling of AN till the 10^{th} day. Afterwards, a decline was observed (Figure 2).

The erythrocytes of fish exposed to 3ppm crude oil (Treatment 1 group) concentration exhibited the highest frequency of AN at the 1^{st} sampling date. After that the trend of appearing of abnormality type was gradually reduced, but the amount of AN was higher than that of the control group at the endpoint of the exposure. However, the fish of the treatment 2 group (15ppm crude oil) revealed a trend of development of altered nuclei in their erythrocytes in a similar way to the control group. Even so, the frequency of AN was highest on each sampling date among the groups [Figure 2].

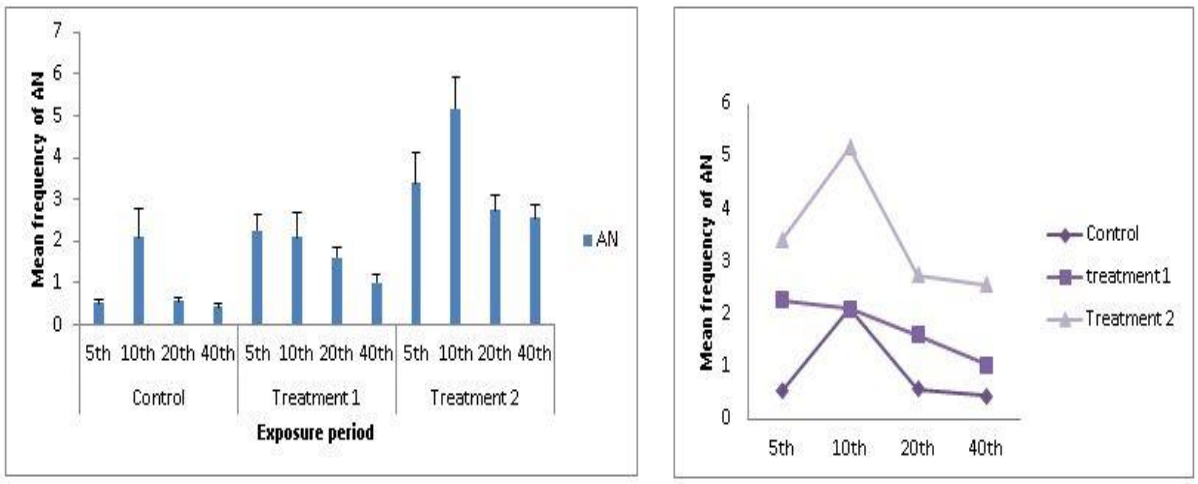

Figure 2. Indication of and trends of altered nuclei (AN), over 40-day exposure period (Mean ± SE calculated from 10 fish per group.)

According to Bolognesi et al., 2006, the application and justification of a sensitive biomarker susceptible to the effects of chemical mixtures is important in bio monitoring aquatic pollution. The present study exhibits the genotoxic effects of sub lethal concentrations of crude oil on *Oreochromis niloticus* peripheral blood. According to the results obtained, there was a significant induction of altered nuclei in peripheral blood which were exposed to crude oil, which has been considered as a reliable approach in assessing the genotoxic effects of certain compounds in crude oil (Ferraro et al., 2004; Hoshina et al., 2008).

When considering about the genotoxic effect of different concentrations of crude oil on fish, the present study showed that there was a significant induction of nuclear abnormalities in fish exposed to a 15ppm concentration of crude oil than in fish exposed to a 3ppm concentration of crude oil. Scientists also discovered this finding for varying amounts of crude oil and other xenobiotic chemicals in fish under controlled settings (Özkan et al., 2011), (Baršienė & Andreikėnaitė, 2007). The observed trend of abnormalities with exposure time also reported by several studies (Gökalp Muranli & Güner, 2011). Scientists argue that fish can generally maintain a consistent concentration of red blood cells under normal conditions through a dynamic equilibrium between fresh erythrocyte production (erythropoiesis) and erythrocyte breakdown, which results in homeostasis. As a result, new erythrocytes enter the circulation at a constant pace, and altered erythrocytes are eliminated at the same rate (Van der Oost et al., 2003). This phenomenon was demarcated with the decreasing trends of AN in all groups after 10^{th} day of exposure. Venier et al. (1996) pointed out same effect, that the treatment with 0.3 and 3 μgl–1 benzo[a]pyrene induced a significant increase in DNA strand breaks in mussel hepatopancreas after one day of exposure, followed by a gradual decrease in strand breaks after 3– 6 days, and after 12 days the frequency of DNA strand breaks returned to the control level. According to that trend, we can argue that the genotoxic effects of fish peripheral blood do not always increase with exposure to crude oil or any other toxic compounds within short term period.

However, there were small number of altered nuclei in the control group throughout the exposure period, but it wasn't a significant amount when compared to exposure groups. Therefore, these findings justify the use of nuclear abnormalities other than micronuclei in fish erythrocytes as a sensitive model for testing the mutagenic activity of chemical substances in the laboratory conditions. Furthermore, the trend suggests that the genotoxic effect of these xenobiotic compounds depends on both time and dose. However, further research is needed to

understand the process of these nuclear abnormalities' development, as well as to study their genotoxic origins.

4. Conclusions

According to the results and observations of the present study, concluding remarks could be kept as the toxic compounds in crude oil are responsible for the development of a significant quantity of altered nuclei in fish erythrocytes with respect to exposure dose and the trends of those abnormalities indicate the association with time of disclosure.

5. References

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