New Horizons Journal of Basic and Applied Sciences, 2026, Vol.X, Iss. X, 144-154

DOI:10.62184/nhjbas.jnh30020271

Research Article

SCIENCE PARK PUBLISHER

New Horizons Journal of Basic and Applied Sciences




# Morphological abnormalities in erythrocytes as a preliminary indicator of genotoxicity of nano-tire rubber in Nile tilapia (*Oreochromis niloticus*)

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**Received: 11, 01, 2026; Accepted: 02, 10, 2026; Published: 3, 11, 2026**

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## Abstract

Despite recent research interest in the toxic effects of tires, there is a lack of data on the genotoxicity of tire rubber particles in aquatic organisms. This study aimed to detect the cellular and genetic abnormalities affecting erythrocytes in Nile tilapia (*Oreochromis niloticus*) exposed to tire rubber nanoparticles (TRNPs ≤1 μm) using a micronucleus assay. Fish were divided into 3 groups: the first (control) was not exposed to TRNPs, the second was exposed to different concentrations of TRNPs in the water (5, 10, and 30 mg/Lw, respectively), and the third was exposed to different concentrations of TRNPs mixed with the feed (100, 150, and 300 mg/kg of the diet, respectively) for a period of 15 days. The frequencies of altered erythrocytes, nuclear lesions, and cytoplasmic abnormalities were significantly lower in the control group compared to those observed in the blood of fish of the exposure groups. In most of these abnormalities, high abundance and statistically significant differences were in the concentrations of the third group, while the general distribution and abundance of these abnormalities were higher in the second group. A direct correlation was found between an increase in these abnormalities and the statistically significant differences increased in a concentration-dependent manner of TRNPs in both groups. These findings provide a preliminary indication of the harmful effects of nano-tire rubber on fish, thereby laying the groundwork for further environmental and biological toxicological research on this pollutant.

**Keywords:** Nano-tire rubber; *Oreochromis niloticus*; Erythrocytes; Genotoxicity; Micronucleus assay

## 1. Introduction

Nowadays, one of the most common environmental contaminants that affect aquatic ecosystems is vehicle tires. Their breakdown results in microscopic particles that enter the water system through surface runoff and harm aquatic life [1-3]. The nanoscale of these particles (≤ 1 μm) is considered the most dangerous to aquatic organisms, as their small size allows them to potentially cause cytotoxic effects [4-6].

Tire particles contain a complex mixture of metals and added organic chemicals, many of which are genotoxic and/or carcinogenic [5, 7]. As these pollutants continue to be produced and released into the aquatic environment, research into their potential genotoxicity to aquatic organisms becomes a priority for scientific research.

Fish genotoxicity is currently considered one of the effective animal models used in assessing the health of aquatic ecosystems [8]. Fish erythrocytes are currently used as a unique tool for assessing cytotoxicity and genotoxicity because they are highly sensitive and respond rapidly to various pollutants, based on the important role of blood in transporting toxins to fish due to direct gill contact with water [9, 10]. Toxic biomarkers in these cells (such as red blood cell morphology, as well as nuclear and cytoplasmic abnormalities) are used as valuable and clear biomarkers for

DOI:10.62184/nhjbas.jnh30020271

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environmental pollution and the toxicity of various chemicals, pharmaceutical residues, ultraviolet radiation, microplastics, stress factors, and others [11-13].

The structure abnormalities test of red blood cells (RBCs) in fish is currently a suitable and effective model and provides a biomarker for cellular and genetic damage on a large scale, as it is simple, sensitive, and fast and doesn't require high technical specifications, as it is performed by only one observer and in a blind manner [14-16].

A common model organism in the study of ecotoxicology is the Nile tilapia, *Oreochromis niloticus* [17]. Exposure to deadly substances has shown several biochemical reactions in this fish [17, 18]. According to [19], this species is an excellent organism for tracking the impacts of xenobiotics.

Although the genomic damage of aquatic organisms exposed to burning tire ash and tire chemical leaching has been studied in recent environmental studies [e.g., 11, 20]. Information remains scarce regarding the genetic effect resulting from tire rubber particles. Therefore, this study aims to document the effect of TRNPs (TRNPs ≤ 1 µm) on cellular, nuclear, and cytoplasmic abnormalities of RBCs in Nile tilapia as initial evidence of genotoxicity.

## 2. Materials and methods

### 2.1. Chemicals

Tire rubber particles with micro-size (<0.5 cm) were purchased from a tire factory (Luxor, Egypt) and converted to nanoscale (≤1 µm) by the hydrothermal liquefaction method according to Chen et al. [21]. These particles were characterized using optical and Scanning Electron Microscopy (SEM) at Assiut University (JSM 5400 LV) according to Mottern [22] (Figure 1).

### 2.2. Fish exposure

A total of 210 adult monosex Nile tilapia (*Oreochromis niloticus*) (mean length and weight 10.2 ± 1.7 cm and 51.1 ± 10.6 g, respectively) was exposed to different concentrations of TRNPs (TRNPs ≤1 µm) in a 15-day experiment after acclimation for another 15 days. The physicochemical properties of the experimental water were water temperature (27.3±0.2 ºC), pH (7.4±0.1), dissolved oxygen (3.05±0.6 mg $L^{-1}$), ammonia (0.7±0.3), and photoperiod (12:12 light:dark), with renewed water regularly daily to remove feces and food scraps by replacing the amount of TRNPs lost from the water tanks according to the water lost. During the experimental period, fish were fed pellet feed that mainly contained 25% crude protein, 5% fat, fiber (4.35%), soybean meal (46%), and fishmeal (65%). By administering the feed twice daily (at 8:00 am and 4:00 pm), a certain quantity of this feed was ground in a feed mill, certain quantities of TRNPs particles were added, and then it was kneaded after adding water and reshaped into balls using a feeding machine. Fish were divided into 3 groups (3 tanks in each concentration × 10 fish per tank). The first group (control) wasn't exposed to TRNPs, the second group was exposed to three varying concentrations of TRNPs in water (5, 10, and 30 mg/Lw, respectively), and the third group was exposed to three varying concentrations of TRNPs mixed with the feed (100, 150, and 300 mg/kg food, respectively). These concentrations were used according to previous research [23].

### 2.3. Analysis of abnormalities of erythrocytes (morphology of RBCs, nuclear, and cytoplasm)

Cellular, nuclear, and cytoplasmic abnormalities in RBCs were analyzed according to [24]. Whole blood was drawn from the caudal vein. Blood smears were placed directly onto clean microscope slides and allowed to air dry for 20 minutes. The smears were fixed with pure methanol for 10 minutes and then stained with 5% Giemsa solution. Ten slides were randomly prepared for each treatment of five fishes, and 1000 cells per slide were recorded under 1000× magnification to determine the frequencies of cells with abnormalities. Coded and randomly generated slides (blind review) were scored by a single observer. The morphology of these abnormalities was quantified according to the criteria of [11].

The frequencies of erythrocyte (E), nuclear (N), and cytoplasmic (C) abnormalities were calculated as follows (Equation (1)):

$$\mathbf{E/\ N\ /\ C\ abnormalities}\ \% = \frac{\textbf{Number of cells containing abnormalities}}{\textbf{Total number of cells counted}} \qquad \textbf{(1)}$$

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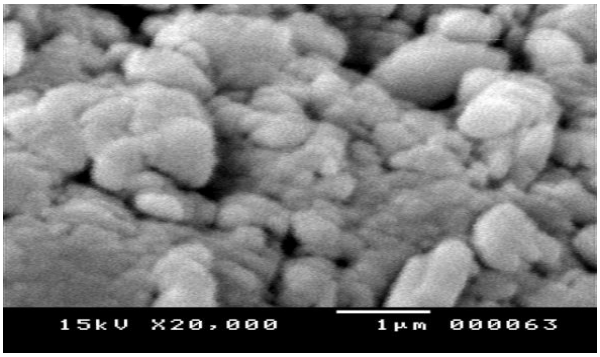



**Figure 1.** Scanning electron micrographs of tire rubber particles after conversion to nanoscale (≤1 µm) showing their aggregation as rough-surfaced, spherical aggregates. Based on the scanning electron micrograph scale (≤1 µm), the estimated particle size ranged between 80 nm and ≤1 µm, with an average size between 0.25 and 0.60 ≤1 µm.

### 2.4. Statistical analysis

A complete database with mean and standard deviations of variables was tabulated and created using Excel software. Data were statistically processed according to [11, 14, 25], as all data were subjected to a Shapiro–Wilk test for normality and Levene's test for homogeneity of variances, utilizing a one-way analysis of variance (ANOVA) test at a $p < 0.05$ level with the SPSS software to evaluate significant differences between the effects of TRNPs on erythrocyte variables among experimental fish groups.

## 3. Results and discussion

### 3.1. Appearance, variation, and numerical frequency of cellular and genetic abnormalities in erythrocytes, nucleus, and cytoplasm of Nile tilapia exposed to TRNPs

The results reveal that RBCs appear normal in the blood of Nile tilapia Oreochromis niloticus in the first group (control), which wasn't exposed to TRNPs. While numerous cellular, nuclear, and cytoplasmic abnormalities were observed in the RBCs of Nile tilapia in the second group, which was exposed to different concentrations of TRNPs in the water (5, 10, and 30 mg/Lw), and the third group, which was exposed to different concentrations of TRNPs mixed with feed (100, 150, and 300 mg/kg feed). These abnormalities include spinocyte, sickle cell, schistocyte, kidney-shaped, acanthocyte, tear-drop shape, microcytic, heart-shaped, eccentric nuclei, crenated cell, and elongated N, as well as nuclear abnormalities, micronuclei, binucleated cells, notched nuclei, lobed nuclei, blebbed nuclei, hook-shaped N, and irregular-shaped N; and cytoplasmic abnormalities, such as vacuolated cytoplasm, degenerated cytoplasm, cytoplasmic granulation, and cytoplasmic shrinkage, whereas these abnormalities were much less pronounced and significantly less noticeable in the first group (Table 1 and Figure 2, 3, and 4).

The third group recorded the highest abundance and the highest statistically significant differences in sickle cell, schistocyte, kidney-shaped, acanthocyte, tear-drop shape, microcytic, heart-shaped, crenated cell, elongated N, micronuclei, binucleated cells, notched nuclei, lobed nuclei, hook-shaped N, irregular-shaped N, vacuolated cytoplasm, degenerated cytoplasm, and cytoplasmic shrinkage, while the second group recorded the highest abundance and the highest statistically significant differences in spinocyte, eccentric nuclei, blebbed nuclei and cytoplasmic granulation.

In both groups (second and third), an increase in the abundance and statistically significant differences of all abnormalities was observed, with increasing the concentration of TRNPs.

Generally, cellular morphological abnormalities of RBCs, nuclei, and cytoplasm (each separately) appeared higher in the concentrations of the second and third groups than in the first group, as abnormalities of RBCs and cytoplasm were higher in the concentrations of the second group, while abnormalities of nuclei, were higher in the concentrations of the third group, as well as in the general proportion of all these abnormalities together, which was higher in the concentrations of the second group (Figure 5A and B).

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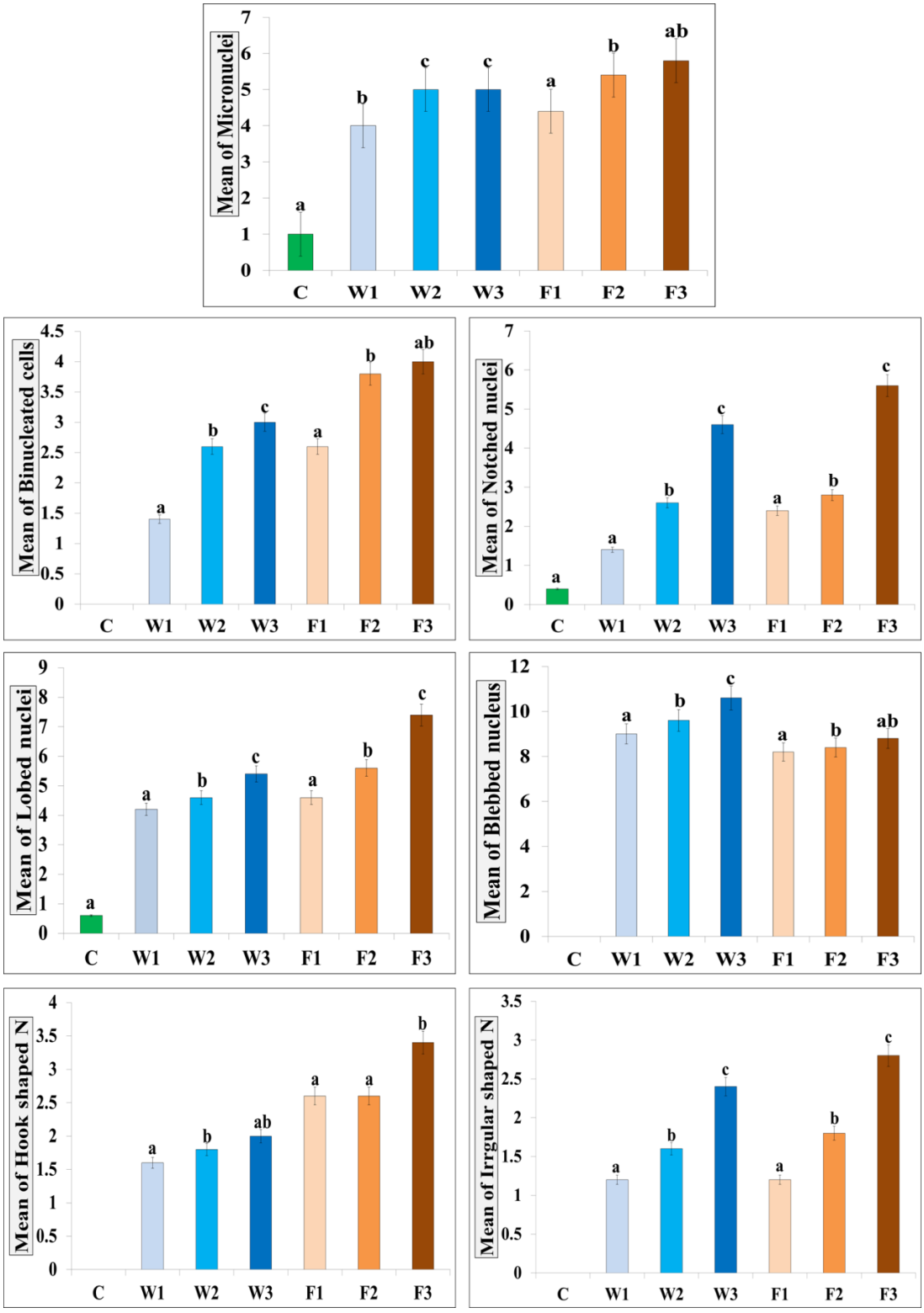



**Figure 2.** Nuclear abnormalities of RBCs in Nile tilapia exposed to different concentrations of TRNPs; C = control, (W1, W2, W3) = different concentrations of TRNPs in water (5, 10, and 30 mg/Lw, respectively), and (F1, F2, F3) = different concentrations of TRNPs in food (100, 150, and 300 mg/kg, respectively). Data are represented as means ± SD. Values with different superscript letters in the same figure for each parameter are significantly different from each other at ($P < 0.05$).

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**Table 1. Mean± SD of cellular abnormalities (%) of RBCs morphology in Nile tilapia, *Oreochromis niloticus*, exposed to different concentrations of nano tires rubber.**

| | Parameters | Control | Water treatments | | | Food treatments | | |
|---|---|---|---|---|---|---|---|---|
| | | | W1 | W2 | W3 | F1 | F2 | F3 |
| Altered erythrocytes | Spinocyte | 0.0 | 33.2±9.6$^{a}$ | 32.8±6.3$^{a}$ | 45.6±8.9$^{b}$ | 4.2±1.5$^{a}$ | 5.6±4.5$^{b}$ | 5.6±2.7$^{b}$ |
| | Sickle cell | 0.0 | 2.2±0.7$^{a}$ | 2.8±1.6$^{a}$ | 3.2±2.1$^{b}$ | 3.4±2.1$^{a}$ | 3.8±2.04$^{b}$ | 4.2±2.1$^{c}$ |
| | Schistocyte | 0.0 | 2.2±0.7$^{a}$ | 3.2±1.8$^{b}$ | 3.4±1.4$^{ab}$ | 2.8±1.3$^{a}$ | 3.2±1.5$^{b}$ | 3.4±2.5$^{ab}$ |
| | Kidney- shaped | 0.2±0.4$^{a}$ | 3±0.6$^{a}$ | 4.2±2.6$^{b}$ | 4.8±2.2$^{ab}$ | 2.8±0.7$^{a}$ | 3.2±1.2$^{b}$ | 3.8±1.2$^{ab}$ |
| | Acanthocyte | 0.0 | 1.4±0.5$^{a}$ | 1.6±0.5$^{a}$ | 2.2±1.2$^{b}$ | 1.8±0.4$^{a}$ | 2.2±0.7$^{b}$ | 4.2±2.9$^{c}$ |
| | Tear-drop shape | 0.0 | 5.8±3.1$^{a}$ | 6±2.4$^{b}$ | 6.2±3.9$^{b}$ | 7.2±3.9$^{a}$ | 9±2.2$^{b}$ | 10±4.05$^{c}$ |
| | Microcytic | 1.6±0.5$^{a}$ | 3.2±1.8$^{b}$ | 6.4±1.4$^{c}$ | 6.8±2.9$^{c}$ | 6.6±2.9$^{a}$ | 7±1.7$^{b}$ | 7.2±1.03$^{ab}$ |
| | Heart- shaped | 0.0 | 1.4±0.8$^{a}$ | 1.4±0.5$^{a}$ | 2.8±0.4$^{b}$ | 1.6±0.8$^{a}$ | 3±1.1$^{b}$ | 3.8±0.4$^{ab}$ |
| | Eccentric nuclei | 1.2±0.4$^{a}$ | 3.4±0.8$^{b}$ | 3.6±1.07$^{b}$ | 4±0.6$^{c}$ | 2.8±1.2$^{a}$ | 3.2±1.5$^{b}$ | 4±1.3$^{c}$ |
| | Crenated cell | 0.4±0.5$^{a}$ | 1±0$^{a}$ | 1.2±0.4$^{b}$ | 1.4±0.5$^{ab}$ | 1.6±0.8$^{a}$ | 1.8±0.4$^{b}$ | 2.4±1.8$^{c}$ |
| | Elongated N | 1.4±1.07$^{a}$ | 4.8±1.03$^{b}$ | 5.2±2.5$^{ab}$ | 5.4±3.02$^{c}$ | 4.8±2.3$^{a}$ | 6.2±3.2$^{b}$ | 8.6±2.4$^{c}$ |

(W1, W2, W3) = different concentrations of TRNPs in water (5, 10, and 30 mg/Lw, respectively), (F1, F2, F3) = different concentrations of TRNPs in food (100, 150, and 300 mg/kg, respectively). Data are represented as means ±SD. Values with different superscript letters in the same row for each parameter are significantly different from each other at (P <0.05).

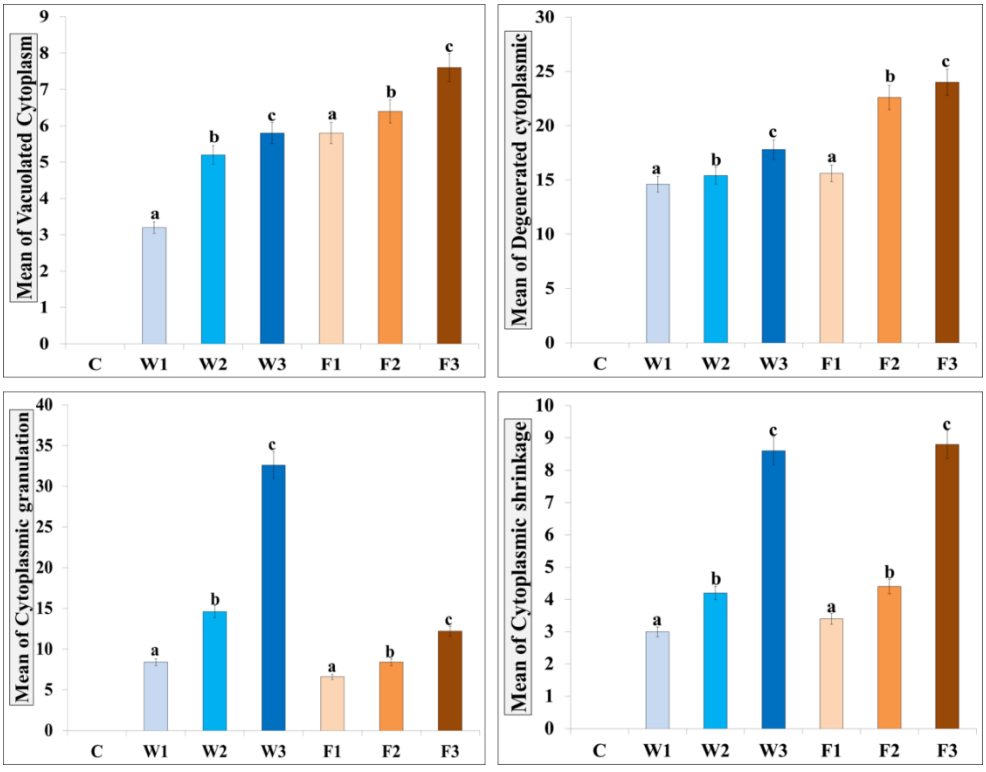



**Figure 3.** Cytoplasm abnormalities of RBCs in Nile tilapia exposed to different concentrations of TRNPs: C = control, (W1, W2, W3) = different concentrations of TRNPs in water (5, 10, and 30 mg/Lw, respectively), and (F1, F2, F3) = different concentrations of TRNPs in food (100, 150, and 300 mg/Kg, respectively). Data are represented as means ± SD. Values with different superscript letters in the same figure for each parameter are significantly different from each other at (P <0.05).

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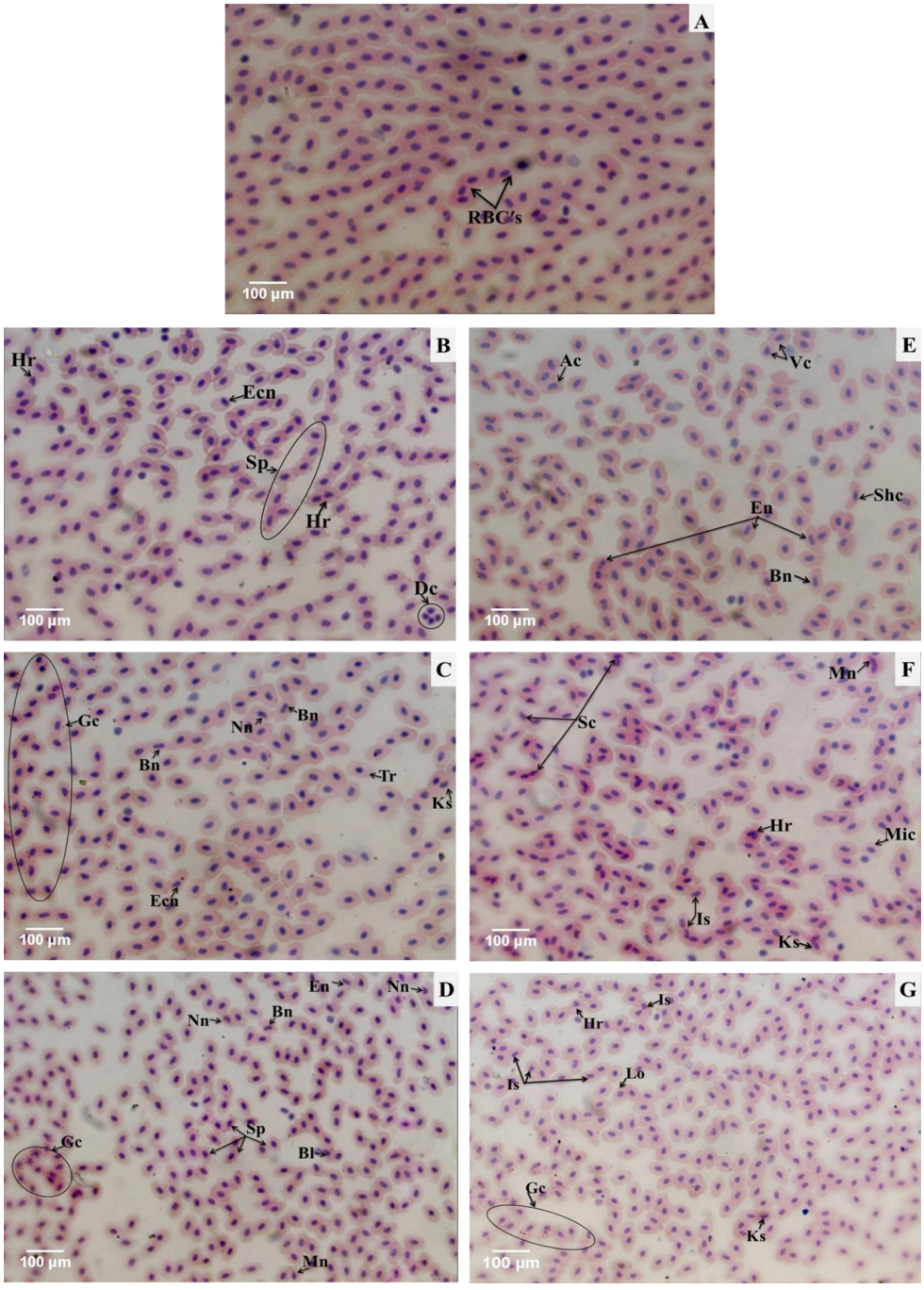



**Figure 4.** Micrograph photos for Giemsa-stained blood smears of Nile tilapia: (A) RBCs of control fish with normal erythrocytes; (B, C, and D) RBCs of fish exposed to different concentration of TRNPs in water; B) 5 mg/Lw, C) 10 mg/Lw, and D) 30 mg/Lw and (E, F, and G) RBCs of fish were exposed to different concentrations of (TRNPs) in food: E) 100 mg/Kg, F) 150 mg/Kg, and G) 300 mg/Kg showing deformed erythrocytes after TRNPs exposure; Erythrocytes with eccentric nucleus (Ecn), teardrop cell (Tr), spinocyte (Sp), acanthocyte (Ac), binucleated RBCs (Bn), micronucleus (Mn), kidney shape (Ks), heart shape (Hr), elongated nucleus (En), blebed nuclei (Bl), lobed nuclei (Lo), microcyte (Mic), schistocytic (Shc), notched nucleus (Nn), hook-shaped nucleus (Hs), irregularly shaped nucleus (Is), degenerated cytoplasm (Dc), vacuolated cytoplasm (Vc), and granulated cytoplasm (Gc) (scale bar: 100 µm).

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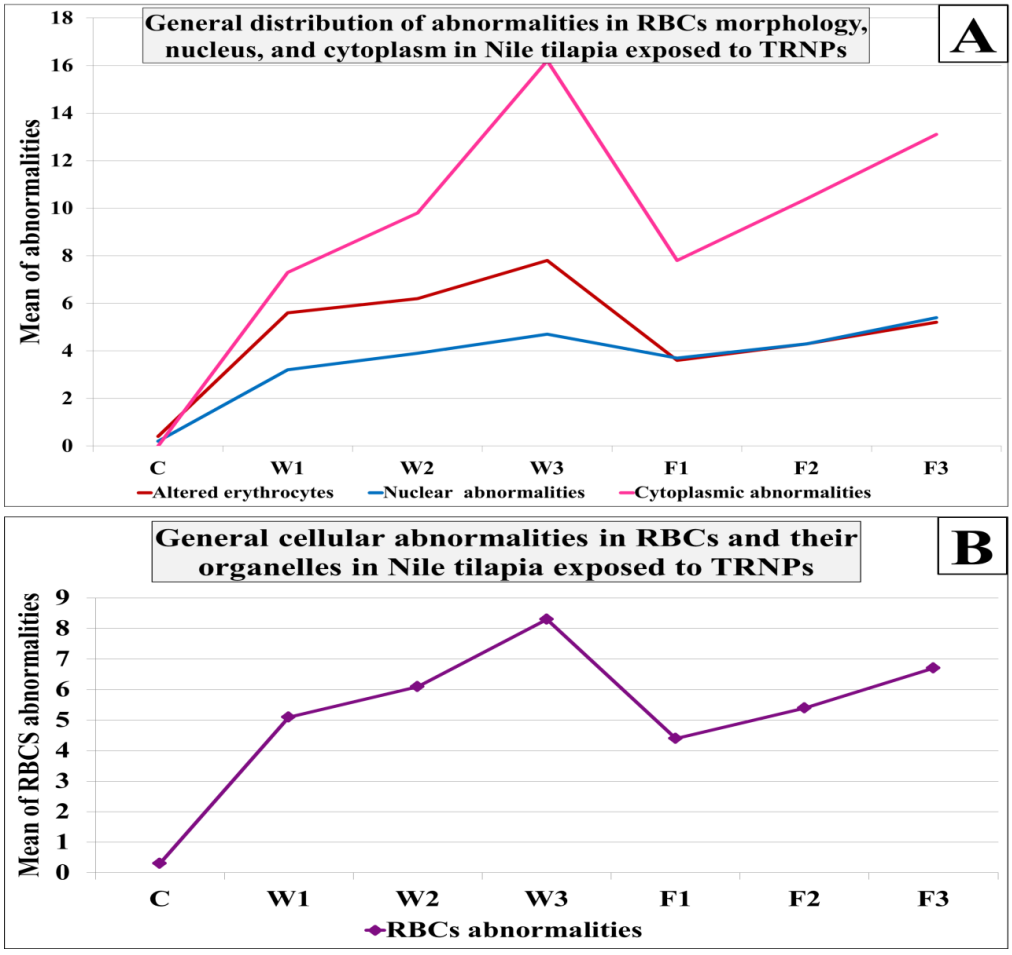



**Figure 5.** A) General distribution of abnormalities of erythrocytes, nuclei, and cytoplasm (each separately) and B) General cellular abnormalities of RBCs and their organelles in Nile tilapia exposed to different concentrations of TRNPs; C = control, (W1, W2, W3) = different concentrations of TRNPs in water (5, 10, and 30 mg/Lw, respectively), and (F1, F2, F3) = different concentrations of TRNPs in food (100, 150, and 300 mg/Kg, respectively).

The current study makes a clear scientific contribution by using a research method that balances technical simplicity with the accuracy of the data presented on the use of cellular abnormalities as preliminary biomarkers for genotoxicity caused by TRNPs (TRNPs ≤1 µm) which results from the use of vehicles that have become essential in today's world. Despite the potential risk posed by this size of targeted pollutant to the cells of aquatic organisms, its impact on genotoxicity in aquatic biota remains poorly understood.

It is now recognized that exposure of aquatic organisms, particularly fish, to environmental pollutants in general causes profound cellular disturbances. According to [14, 25], these disturbances manifest in changes in the morphology of cells and their organelles and negatively affect their integrity and the performance of their vital functions. This was evident in our results, which were represented by a marked increase and diversity in the average number of abnormal RBCs with asymmetrical shapes, such as spinocyte, sickle cell, schistocyte, kidney-shaped, acanthocyte, tear-drop-shaped, microcytic, heart-shaped, eccentric nuclei, crenated cell, and elongated N, as well as nuclear abnormalities, micronuclei, binucleated cells, notched nuclei, lobed nuclei, blebbed nuclei, hook-shaped N, and irregularly shaped N, and cytoplasmic abnormalities, such as vacuolated cytoplasm, degenerated cytoplasm, cytoplasmic granulation, and cytoplasmic shrinkage. These abnormalities were found in the blood of Nile tilapia, *Oreochromis niloticus* exposed to varying

DOI:10.62184/nhjbas.jnh30020271



concentrations of TRNPs in the water and mixed with the feed, compared to the blood of the control fish, which wasn't exposed to TRNPs, indicating the possibility of acute genotoxicity in fish exposed to TRNPs (regardless of the method of exposure).

Oxidative stress resulting from the toxic effects of tire components can cause these deformities, which aligns with [20, 26, 27], who indicated that tires contain a mixture of known and unknown chemicals, in addition to heavy metals, tire rubber, and others, leading to oxidative stress that causes deformities in RBCs. Tire particles may also interfere with overall cellular metabolic activity, potentially leading to cytotoxicity and the appearance of these abnormalities [28, 29]. Changes in cell division rates are harmful, as they can disrupt vital processes such as gene transcription and DNA replication, potentially leading to gene mutations, chromosomal abnormalities, and ultimately cell death [11, 30, 31]. Our findings are consistent with those of [11], who reported that exposure to burning tire ash increases oxidative stress, cell damage, and genotoxicity (nuclear and cellular abnormalities in RBCs) in exposed fish. Although the types of fish targeted in the two studies differed, the abundance and diversity of these cellular abnormalities in our study may be attributed to the different components of the targeted tires: our study targeted TRNPs containing carbon black, chemicals, and aromatic hydrocarbons [32, 33], while [11] targeted burnt car tire ash.

Similarly, our study shows clear agreement in the occurrence of structural abnormalities in RBCs with other studies targeting different types of emerging environmental pollutants and other freshwater fish species [e.g., 12, 14, 25]. However, the increased abundance and remarkable diversity of these abnormalities in our study also indicate a higher risk of concentration of the targeted pollutant and its effect on the targeted fish species compared to the aforementioned studies.

This is according to [34, 35]**,** who indicated that tire rubber is a type of plastic, considering the nanoscale size targeted in our study compared to the microscale size (<5 mm) of other plastic types targeted in other studies of plastics and other materials [12, 14, 25, 36]. Recent scientific studies indicate that nanoparticles have the ability to penetrate cells and have a greater impact, causing significant damage to the DNA of blood and brain cells [22, 37]. Compared to the results of studies targeting nanoscale particles of other plastic pollutants, we find an increase and diversity in the genetic abnormalities occurring in fish exposed to tire rubber in our study, which may be due to an increase in the concentration or type of the pollutant [38].

Although the group exposed to varying concentrations of TRNPs mixed with feed exhibited a higher abundance of most abnormalities in the blood of *Oreochromis niloticus* compared to other groups, which may be attributed to the different route the pollutant enters the fish's body. The first route involves the pollutant entering the body through ingestion during feeding, while the second relies on entering via the gills during respiration. This aligns with the findings of [39, 40], who identified these two routes of ingestion in fish in nature; however, the overall increase in the abundance of malformations (cellular, nuclear, and cytoplasmic) individually, as well as the combined increase in all these malformations, in the group exposed to varying concentrations of TRNPs in the water compared to other groups, suggests the significant impact of this targeted pollutant on the blood of *Oreochromis niloticus* (regardless of the route of exposure).

Generally, the increased rate of abnormalities in RBCs and the greater presence of micronuclei with the increasing of concentration and duration, seem logical and consistent with other studies targeting the same fish species in our study [e.g., 13, 25]. Increased abnormalities may lead to cancer in organisms and cause a decrease in red blood cell count (anemia) [13].

## 4. Conclusion

This study provides clear evidence of nuclear and cellular abnormalities as early indicators of genotoxicity caused by fish exposure to nano-tire rubber via various routes, raising concerns about this pollutant and calling for further research to confirm its toxicity using DNA damage assays. The study also recommends further research into the environmental and biological toxicity of this pollutant.

## Authors' contributions

Osman, A. G. M., Mahdy, A., and Badrey, A. E. A. provided the research idea. Hassan, Y. A. M. and Badrey, A. E. A. designed and conducted the experiment, analyzed and interpreted the data, and prepared the initial draft of the manuscript. All authors contributed significantly to critical

DOI:10.62184/nhjbas.jnh30020271

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review of the scientific content and final revision of the manuscript.

## Conflict of interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Funding

No funding was received for writing this manuscript.

## Ethics approval

Experimental setup and fish handling were approved by the Research Ethics Committee of the Faculty of Science, Al-Azhar University, Assuit, Egypt. AZHAR 24/2025.

## Code Availability

It is not applicable.

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## Data Availability

All data generated or analyzed during this study are included in the research article.

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DOI:10.62184/nhjbas.jnh30020271

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