Trop J Nat Prod Res, February 2024; 8(2):6300-6303 ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

The Effect of Exposure to Polystyrene Nanoplastics on Cytokine Levels and Reproductive System of Male Tilapia

Alfiah Hayati[1]*, Widi P. Wilujeng[1], Manikya Pramudya[1], Firli R.P. Dewi[1], Sugiharto[1], Bayyinatul Muchtaromah[2], Raden J.K. Susilo[3]

[1]*Department of Biology, Faculty of Science and Technology, Universitas Airlangga, Surabaya 60115, Indonesia*
[2]*Department of Biology, Faculty of Science and Technology, Universitas Islam Negeri Maulana Malik Ibrahim, Malang 65114, Indonesia*
[3]*Nanotechnology Engineering, Faculty of Advance Technology and Multidiscipline, Universitas Airlangga, Surabaya 60115, Indonesia*

ARTICLE INFO

Article history:
Received 21 January 2024
Revised 14 February 2024
Accepted 19 February 2024
Published online 01 March 2024

ABSTRACT

Several studies demonstrated adverse effects associated with exposure to polystyrene nanoplastics (NPs), such as toxicity, inflammation, or other health-related concerns. This study aims to analyse the impact of NPs exposure on pro-inflammatory cytokine levels and testicular histology in tilapia gonads. Twenty-four male tilapias were used in this study and divided into four groups, including a control (commercial pellets only) and three treatment groups (commercial feed mixed 0.5, 1.0 and 2.0 µL/kg of NPs, respectively) for 25 days. Blood sample was used to tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) levels analysis. Histology of testicular tissue was prepared with hematoxylin and eosin (HE) staining. The results showed that NPs exposure has no significant influence of TNF-α and IFN-γ levels. The levels of TNF-α on the control (1.6 ± 0.3 ng/mL) and the treatment groups from low to high concentration (1.7± 0.4; 1.7 ± 0.4; and 1.6 ± 0.3 ng/mL) and the levels of IFN-γ control (16.7 ± 2.8 ng/mL) and the treatment groups (19.1 ± 5.3; 18.7 ± 4.2; and 19.8 ± 3.5 ng/mL). Polystyrene nanoplastics exposure can reduce the number of spermatogenic cells. Spermatogonia cells was decreased from 27.5 ± 0.9 to 13.1 ± 1.1 cells/cyst. Spermatocyte cell was decreased from 57.6 ± 0.6 to 18.5 ± 0.3 cells/cyst. Spermatid cells was decreased from 86.8 ± 2.7 to 38.5 ± 2.7. In conclusion, exposure to multiple doses of NPs for 25 days did not increase pro-inflammatory cytokines levels in the blood serum of tilapia, but it caused histological changes in the gonads of fish.

Keywords: Tilapia, Nanoplastics, Cytokine, Gonad, Healthy life, Spermatogenic.

Introduction

Polystyrene nanoplastics (NPs) is often use by several people for industrial and commercial activities.[1] Primary micro-nano plastics are plastic particles that are directly released into environment such as microfibers, fragments, microbeads, and plastic pellets.[2] Secondary microplastics was originate from degradation of larger plastic products after exposure to environment with regular or irregular shapes formed from abrasion processes, mechanical wear as wave action, photo-oxidation, and biological degradation down to NPs size.[3] Furthermore, contamination become very extensive and cannot be degraded naturally in nature because of its nano size and accumulate in aquatic organism and disturb its metabolism.[4] Long-term exposure to NPs increase oxidative stress which lead to tissue damage in several organs.[5] In general, NPs can enter body through digestion and respiration processes.[6] The abundance of NPs particles in water raise concern about the potential toxicity for aquatic organism including fish that are often consumed by human.[7] In previous study, polystyrene microplastics can impair gonad of zebrafish which lead to apoptosis.[8] Furthermore, NPs have high possibility to affect gonad of fish due to microplastic can still be broken down again become nanoscale.[9,10]

*Corresponding author. E mail: alfiah-h@fst.unair.ac.id
Tel: +62 813-3095-0399

Citation: Hayati A, Wilujeng WP, Pramudya M, Dewi FRP, Sugiharto, Muchtaromah B, Susilo RJK. The Effect of Exposure to Polystyrene Nanoplastics on Cytokine Levels and Reproductive System of Male Tilapia. Trop J Nat Prod Res. 2024; 8(2):6300-6304. http://www.doi.org/10.26538/tjnpr/v8i2.30

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria.

Oreochromis niloticus (tilapia) is one of freshwater fish which native from tilapia River basin and several other African river systems.[11] This fish has desirable characteristics and ability to adapt in various environments and widely cultivated in freshwater around the world.[12] Moreover, tilapia has high reproductivity which so benefit for aquaculture.[13] It is very necessary to observe the toxic impact of NPs contamination that accumulates in body of tilapia. Observation of NPs toxicity on pro-inflammatory cytokine levels and reproductive system of tilapia is still inadequate. Pro-inflammatory cytokine is so important as key indicator for inflammation which impair fish metabolism.[14] Meanwhile, several studies were still had lack of specific information regarding the relationship between reproductive system and reproductive activity in terms of spermatogenesis and histology of testis after NPs exposure.[15] The research aims to analyze several NPs concentrations effect on pro-inflammatory cytokine levels such as tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) levels and reproductive system of tilapia gonads.

Matrials and Methods

Animal and Ethical Clearance

Twenty-four male tilapia, 3-4 months old, and weighing 150 ± 0.5 g obtained from the Umbulan Freshwater Aquaculture Development Technical Implementation Unit, Pasuruan, East Java, Indonesia. Experimental fish were performed ethically according to international guidelines and have received an ethical certificate from Health Research Ethical Clearance Commission, Faculty of Dental Medicine, Universitas Airlangga (715/HRECC.FODM/IX/2022). Animal care of tilapia was always observed during the study (28-30°C, pH 7-8, 12:12 h light/dark cycle). Fish were treated in an aquarium connected to water filter pump (Armada-Aquarium Top Filter, Color Aquatic Indonesia) and aerator (Amara-AA350, co. MTA-4362145-Indonesia).

Commercial pellet feed (Comfeed, Aqua Feed-Japfa, PT Suri Tani Pemuka Indonesia) as much as 2-3% of the fish weight was given twice/day for 25 days.

Experimental Design
Fish were acclimated for two weeks at the Fish Maintenance Laboratory, Universitas Airlangga. Twenty-four tilapias were divided into four treatments with six replications and NPs (Sigma Aldrich St. Louis, USA) exposure done for 25 days (Table 1). NPs were sprayed on pellet feed and dried before it was given to fish. Fish was rendered unconsciously by inducing of 0.1 mL/L clove oil containing eugenol before sacrificed. Clove oil was used to calm the fish for a few minutes without any rejection reaction in the fish.

Enzyme Linked Immunosorbent Assay for TNF-*α and* IFN-*γ*
After 25 days of NPs exposure, blood samples were collected for analysis. Tilapia blood was collected through the caudal vein using a 3 mL syringe (Onemed, PT. Jayamas Medica Industri Tbk, Indonesia). Whole blood was collected in 1.5 mL microtubes (Eppendorf, Hamburg-Germany) and centrifuged (Eppendorf 5424R) at 2000 rpm in 4 °C for 10 minutes. The top layer containing serum was collected. The levels of TNF-*α* and IFN-*γ* were measured using the sandwich-ELISA kit (Bioassay Technology Laboratory, Shanghai, China) according to the manufacturer's protocol.

Histological Analysis of Testicular Samples
Tilapia testicular tissue was collected for histopathological analysis. All testis samples from each treatment were collected and fixed in 10% neutral buffer formalin solution. After 24 hours of fixation, the testicular tissue was prepared for paraffin-embedded block sectioning. Approximately 4 µm thickness of each tissue was sectioned with a semi-automatic microtome (Leica RM2125, China). The tissue samples on slide glass were deparaffinized and stained with hematoxylin and eosin (HE). Furthermore, testicular preparations were analyzed under a light microscope (Olympus CX33, Japan). Histological changes were assessed qualitatively and quantitatively. Qualitative observations were made by comparing the control and treatment groups. Meanwhile, quantitative analysis was performed by measuring the diameter of the seminiferous tubules and cysts and counting the number of spermatogenic cells (spermatogonia, spermatocytes, and spermatids) using a digital camera microscope (Optilab, Indonesia) connected to an image raster application.

Statistical analysis
All of data were analysed using the SPSS program version 24 (IBM Corp, USA). One way analysis of variance (each test being conducted at 0.05% level of probability) was used to assess statistical differences between the control and NPs exposure groups. Normality test with Shapiro-Wilk and Levene test for homogeneity. One-way ANOVA variant test ($\alpha = 0.05$) to find out the average difference between the sampling locations and Tukey follow-up test. Histological structure data of tilapia gonads were observed descriptively by presenting them in pictures.

Table 1: Experimental design

No.	Group	NPs Concentration (µL/kg)
1	NPs 0	0
2	NPs 0.5	0.5
3	NPs 1	1.0
4	NPs 2	2.0

Results and Discussion

The Effect of NPs Exposure to TNF-*α and* IFN-*γ Levels*
As shown at Figure 1, after 25 days of exposure to NPs, the levels of TNF-*α* and IFN-*γ* were not significantly ($P>0.05$) different in each group. The results of One-Way ANOVA test showed TNF-*α* and IFN-*γ* had 0.876 and 0.617, respectively. However, the result was still displayed slightly increasing in TNF-*α* and IFN-*γ* levels. The levels of TNF- α on control group (1.6 ± 0.3 ng/mL) and treatment groups from low to high concentration (1.7± 0.4; 1.7 ± 0.4 and 1.6 ± 0.3 ng/mL, respectively). Meanwhile, IFN-*γ* levels on control group (16.7 ± 2.8 ng/mL) and treatment groups (19.1 ± 5.3; 18.7 ± 4.2; and 19.8 ± 3.5 ng/mL, respectively). Polystyrene nanoplastics can pass through the phospholipid membrane layer via endocytosis into cytoplasm, which can enhance cellular cytolytic activity.[16] In this study, the cytotoxic effect of NPs did not affect TNF-*α* and IFN-*γ* levels. A previous study revealed that expression of TNF-*α* and MDA levels were significantly increased in intestines of zebrafish exposed to NPs.[17] However, another factor can affect metabolism in fish beside genetic such as fish size and food concentration.[18] The NPs concentration is still low in this study which not cause an immune response. Nevertheless, these results are similar to other studies which state that NPs cannot directly stimulate pro-inflammatory cytokines.[19] The pro-inflammatory cytokine such as TNF-*α* and IFN-*γ* play an important role in maintaining the dynamic balance of immune responses and are considered effective markers for assessing inflammatory responses.[20-22]

The Effect of NPs Exposure to Testis Histology
The observation of tilapia's testis histology was performed following HE staining. It showed that the shape and number of spermatogenic cells in tilapia testes following treatment with NPs. In NPs 0 group, the number of spermatogenic cells (spermatogonium, spermatocytes, and spermatids) was higher compared to the group exposed to several concentrations of NPs (0.5-2 µL/kg NPs). This condition indicated that NPs were able to enter the tilapia testes and interfere with spermatogenesis process (Figure 2). As shown in Table 2 and Table 3, exposure of NPs significantly ($P<0.05$) reduced the number of spermatogenic and cyst in seminiferous tubules. In addition, diameter of seminiferous tubules and cysts were significantly ($P<0.05$) decreased following exposure to NPs in all concentration. Table 2 showed that NPs exposure could decrease the number of spermatogenic and cysts when compared with data control groups (NPs 0). The number of spermatogonium cells decreased from 27.3 ± 1 to 13.1 ± 1.1 cell. Spermatocyte count was decreased from 57.6 ± 0,6 to 18.5 ± 0.3 cell as same as spermatids number from 86.8 ± 2.7 to 38.5 ± 2.7 cell, the number of cysts in seminiferous tubule was decreased from 8.6 ± 0.36 to 5.9 ± 0.17 cyst. Table 3 showed that NPs was also decreased on diameter of and cyst diameter, when compared with the control group (NPs 0). The diameter of seminiferous tubule displayed 869.1 ± 27.58 to 385.5 ± 11.2 µm and Cyst diameter from 199.1 ± 0.6 to 121.3 ± 0.2 µm.

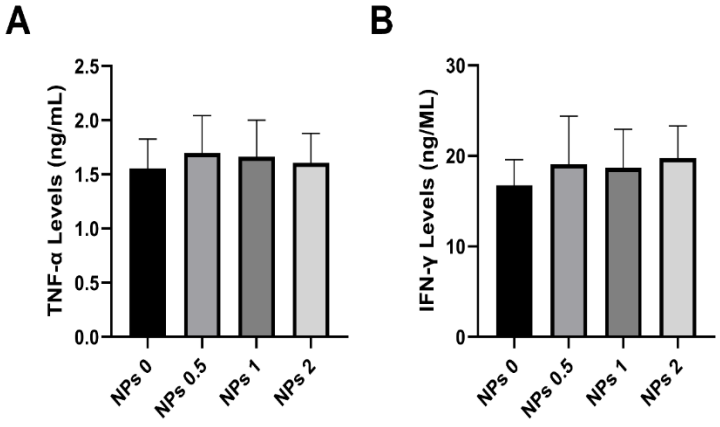

Figure 1: Pro-inflammatory cytokine levels after NPs treatments. (A) TNF-*α* levels; (B) IFN-ɣ levels. TNF-*α*: Tumor necrosis factor- α; IFN-*γ*: Interferon-γ. All of data were displayed by mean ± SD ($\alpha = 0.05$)

Polystyrene nanoplastics as pro-oxidants could elevate ROS that stimulate oxidative stress and activate apoptosis pathways.[8]

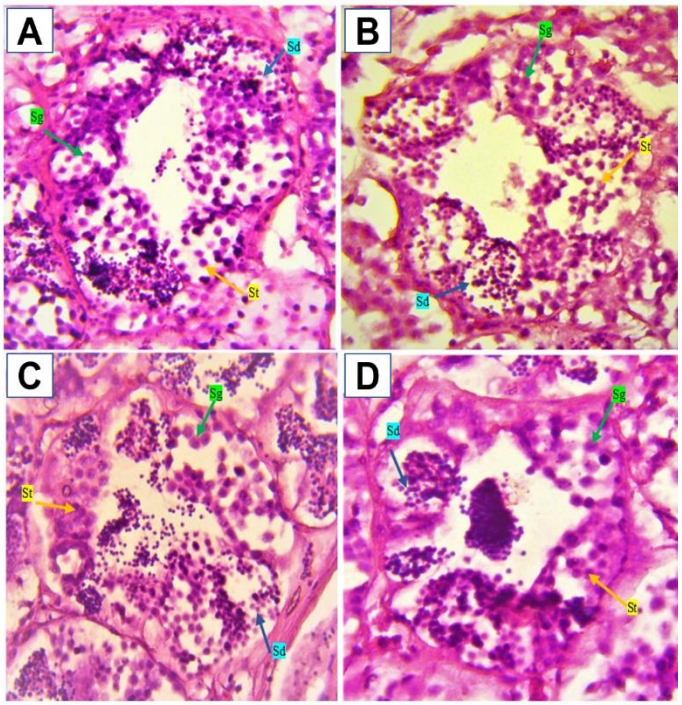

Figure 2: Histology of tilapia's testes after exposure to NPs for 25 days A= control 0 µL/kg NPs; B= 0.5 µL/kg NPs; C= 1 µL/kg NPs; D = 2 µL/kg NPs. Sg: Spermatogonia; St: Spermatocytes; Sd: Spermatids

Furthermore, apoptosis can decrease the number of cells in testicular tissue.[23] In addition, ROS can stimulate lipid peroxidation through polysaturated fatty acid oxidation (PUFA).[24] In this study, exposure with NPs in low concentration to high concentration can affect testicular tissue that undergo decreasing in number of spermatogonium, spermatocytes, and spermatid as same as number of cysts due to oxidative stress. Other studies using zebrafish show the same thing that NPs exposure inhibits the process of spermatogenesis.[25] The NPs exposure reduced number of cysts in seminiferous tubules due to inhibition of spermatogenesis in fish. This condition affects spermatogonium proliferation followed by decreasing of cyst formation.[26,27] There were reducing in diameters of seminiferous tubules and cyst after exposed with NPs concentrations due to oxidative stress in body.[28] Several studies are also state that oxidative stress can stimulate reducing in diameter and inhibition of spermatogenesis.[29-31] Moreover, chronic oxidative stress stimulates low performance in reproductive activity which reduce thickness of seminiferous tubule epithelium.[32-35]

Conclusion

In conclusion, while multiple doses of NPs for 25 days did not exhibit a significant increase in TNF-α and IFN-γ levels, only slight elevations were observed. However, NPs exposure resulted in notable damage to gonadal histology, alterations in the number of spermatogenic cells and cysts, and changes in diameter of seminiferous tubules and cysts. These findings suggest the necessity for further research to assess enzymatic pathways for apoptosis, and it is also intriguing to investigate mRNA expression in future studies.

Conflict of Interest

The authors declare no conflict of interest.

Table 2: The number of spermatogenic and cyst after NPs exposure

Treatments	Spermatogenic count (cell)			Cyst count (cyst)
	Spermatogonium	Spermatocyte	Spermatids	
NPs 0	27.3 ± 1	57.6 ± 0.6	86.8 ± 2.7	8.60 ± 0.36
NPs 0.5	17.1 ± 0.7****	36.4 ± 0.3****	63.4 ± 2.4****	7.17 ± 0.15****
NPs 1	13.7 ± 0.7****	26.4 ± 0.4****	50.4 ± 0.8****	6.77 ± 0.08****
NPs 2	13.1 ± 1.1****	18.5 ± 0.3****	38.5 ± 2.7****	5.90 ± 0.17****

All of data were displayed by mean ± SD (α = 0.05). ****P<0.0001 compared with NPs 0 group

Table 3: Diameter of seminiferous tubules and cysts after NPs exposure

Treatments	Diameter (µm)	
	Seminiferous tubule	Cyst
NPs 0	869.13 ± 27.6	199.10 ± 0.6
NPs 0.5	708.83 ± 2.8****	167.60 ± 1.1****
NPs 1	464.73 ± 3.3****	125.40 ± 0.8****
NPs 2	385.47 ± 11.2****	121.27 ± 0.2****

All of data were displayed by mean ± SD (α = 0.05). ****P<0.0001 compared with NPs 0 group

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

Acknowledgements

The authors give appreciation to the Universitas Airlangga for funding this research through Faculty Research Excellence, the fiscal year 2022 (66/UN3.15/PT/2022).

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