Enteric neuronal density of *Danio rerio* modulated by exposure to textile microfibers and ibuprofen

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Abstract: Pollution by emerging contaminants, such as synthetic microfibers and pharmaceuticals, threatens aquatic biota, including the enteric nervous system of *Danio rerio*. This study evaluated the effects of polyester microfibers, alone or combined with ibuprofen, on enteric neuronal density. Wholemount preparations of the intestinal muscular tunic were subjected to anti-HuC/D immunofluorescence. There was a trend toward reduced neuronal density with microfibers and increased density with ibuprofen, with a significant difference only between Polyester and Aged Polyester + Ibuprofen (Dunn, $p < 0.05$). The results indicate a modulatory effect of ibuprofen on the enteric neuronal response.
Keywords: Enteric nervous system; emerging contaminants; neurotoxicity; myenteric plexus.

Densidade neuronal entérica de *Danio rerio* modulada pela exposição a microfibras têxteis e ibuprofeno

Resumo: A poluição por contaminantes emergentes, como microfibras sintéticas e fármacos, ameaça a biota aquática, incluindo o sistema nervoso entérico de *Danio rerio*. Este estudo avaliou os efeitos de microfibras de poliéster, isoladas ou combinadas com ibuprofeno, sobre a densidade neuronal entérica. Preparações de montagem total (whole-mount) da túnica muscular intestinal foram submetidas à imunofluorescência para anti-HuC/D. Observou-se uma tendência de redução da densidade neuronal com as microfibras e de aumento com o ibuprofeno, com diferença significativa apenas entre os grupos Poliéster e Poliéster Envelhecido + Ibuprofeno (Dunn, $p < 0,05$). Os resultados indicam um efeito modulador do ibuprofeno sobre a resposta neuronal entérica.
Palavras-chave: Sistema nervoso entérico; contaminantes emergentes; neurotoxicidade; plexo mioentérico.

Introduction

The continuous release of xenobiotic compounds into aquatic ecosystems has consolidated emerging contaminants (ECs) as an important environmental challenge due to their persistence, low removal by conventional treatments, and potential to cause biological effects even at low environmental concentrations. The simultaneous occurrence of these pollutants results in complex exposure scenarios, with possible additive, synergistic, or antagonistic interactions [1–4]. Among ECs, nonsteroidal anti-inflammatory drugs (NSAIDs), such as ibuprofen, and polyester microfibers, one of the main forms of microplastic of textile

origin, stand out; these can act as adsorption surfaces and transport vectors for contaminants [5–9]. In addition, environmental weathering, resulting from factors such as UV radiation, mechanical abrasion, and microbial degradation, promotes the aging of these microfibers, altering their physicochemical properties and potentially increasing their capacity to adsorb organic compounds [10]. As the gastrointestinal tract (GIT) constitutes an important route of exposure to ingested contaminants and harbors the enteric nervous system (ENS), which is involved in controlling motility, secretions, and epithelial barrier integrity, investigating the isolated and combined effects of ibuprofen and virgin and aged polyester microfibers in *Danio rerio* allows a realistic understanding of the impacts of these contaminants on intestinal homeostasis [11,12].

Objectives

The present work aimed to evaluate the effects of virgin and aged polyester microfibers, alone or with previously adsorbed ibuprofen, on the enteric neuronal density of *Danio rerio*.

Material and Methods

Adult male *Danio rerio* were exposed for 21 days to six experimental groups, in triplicate, with 40 fish per replicate: control (C); ibuprofen (IBU; 3 μg L^{-1}); virgin polyester microfibers (P; 531 MFs L^{-1}), aged polyester microfibers (PE; 531 MFs L^{-1}), virgin polyester microfibers + ibuprofen (PIBU; 531 MFs L^{-1} + 3 μg L^{-1}); and aged microfibers + ibuprofen (PEIBU; 531 MFs L^{-1} + 3 μg L^{-1}). The microfiber concentration was defined based on values reported for wastewater treatment plant effluents, while the ibuprofen concentration was selected based on environmentally relevant concentrations reported in surface waters receiving wastewater treatment plant effluents [13]. During exposure, physicochemical water parameters and mortality were monitored, using blank controls containing filtered water to evaluate microfiber deposition [14]. At the end of the experimental period, fish were euthanized with benzocaine, and the intestines were collected, washed in 0.1 M PBS (pH 7.4), filled and fixed in 4% paraformaldehyde (12 h at 4 °C). Subsequently, they were washed in PBS/DMSO and stored in PBS containing 0.1% sodium azide. Wholemount preparations of the intestinal muscular tunic were obtained under a stereomicroscope and subjected to immunofluorescence for labeling HuC/D-immunoreactive neurons (HuC/D-IR). Tissues were permeabilized in PBS/Triton X-100 0.5%, blocked in the same permeabilization solution supplemented with 4% BSA, and incubated for 72 h with primary anti-HuC/D antibody (Invitrogen, A-21271). Then, they were washed again in PBS/Triton X-100 0.5% and incubated for 1 h with Alexa Fluor 488 secondary antibody (Abcam, ab150113) and mounted in SlowFade™ Glass (Thermo Fisher).

The density of HuC/D-IR neurons (neurons/mm²) was quantified under a BX51 fluorescence microscope (Olympus), at 40x magnification, using the Motic Image® system. Twenty fields per animal were analyzed in the mesenteric and antimesenteric regions.

Results

Figure 1 presents the density of HuC/D-immunoreactive neurons (HuC/D-IR) in the intestine of Danio rerio after the exposure period. The lowest neuronal density was observed in the polyester microfiber group (P), representing an approximately 40% reduction compared with the control group, while the aged polyester microfiber group (PE) showed a reduction of approximately 21%. In contrast, exposure to ibuprofen alone (IBU) increased neuronal density by approximately 15% relative to the control. Fish exposed to polyester microfibers combined with ibuprofen (PIBU) exhibited an increase of approximately 41%, whereas the aged polyester microfiber plus ibuprofen group (PAPIBU) showed the highest neuronal density, with an increase of approximately 76% compared with the control group. Significant differences among experimental groups were detected by the Kruskal–Wallis test followed by Dunn's post hoc test ($p < 0.05$).

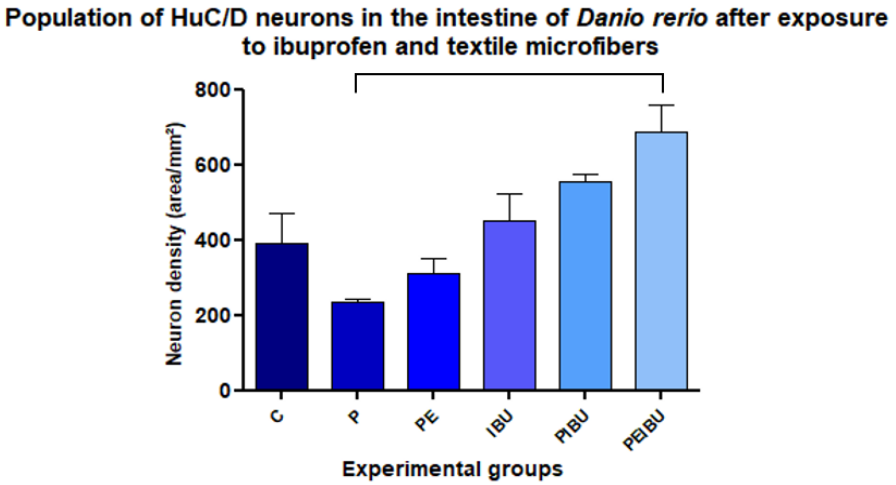


Figure 1. Density of HuC/D-immunoreactive neurons (HuC/D-IR) in the intestine of *Danio rerio* after exposure to ibuprofen and virgin and aged polyester microfibers, alone or combined with the drug. Data are presented as mean ± standard deviation. Differences among groups were evaluated by the Kruskal–Wallis test followed by Dunn's post-test, with *p* < 0.05.

Compared with the control group, fish exposed exclusively to virgin polyester microfibers (P) showed approximately 40% lower HuC/D-IR neuronal density, while the aged polyester microfiber group (PE) exhibited approximately 21% lower neuronal density. In contrast, groups exposed to ibuprofen showed higher neuronal densities than the control group, with increases of approximately 15% in the IBU group, 41% in the PIBU group, and 76% in

the PEIBU group. Dunn's post hoc test identified a significant difference only between the Polyester (P) group and the Aged Polyester + Ibuprofen (PEIBU) group ($p < 0.05$). Overall, these findings indicate that exposure to polyester microfibers alone was associated with lower HuC/D-IR neuronal density than the control, whereas treatments containing ibuprofen were associated with higher neuronal density, suggesting that ibuprofen may modulate the enteric neuronal response under co-exposure conditions.

Discussion

The density of HuC/D-immunoreactive enteric neurons is a relevant parameter for investigating alterations in the enteric nervous system (ENS) following exposure to emerging contaminants. In the present study, fish exposed exclusively to virgin (P) and aged (PE) polyester microfibers presented lower HuC/D-IR neuronal density than the control group. Although these differences were not statistically significant, this pattern resembles responses reported in studies investigating the effects of microplastics and synthetic microfibers on the gastrointestinal tract, including tissue irritation, inflammatory processes, and oxidative stress [7,8]. Furthermore, environmental weathering can modify the physicochemical properties of polyester microfibers, altering their surface characteristics, chemical composition, and interactions with organisms and associated contaminants [10,14].

In contrast, fish exposed to ibuprofen alone (IBU) and to co-exposure treatments (PIBU and PEIBU) presented higher HuC/D-IR neuronal density than the control group. Previous studies have shown that anti-inflammatory interventions may influence enteric neuronal homeostasis under inflammatory conditions [15]. However, these findings were obtained in different experimental models and cannot be directly extrapolated to ibuprofen exposure in *Danio rerio*. Therefore, the higher neuronal density observed in the present study should not be interpreted as evidence of a neuroprotective effect of ibuprofen.

Dunn's post hoc test identified a significant difference only between the Polyester (P) and Aged Polyester + Ibuprofen (PEIBU) groups ($p < 0.05$). The PEIBU group exhibited significantly higher HuC/D-IR neuronal density than the P group. Because these treatments differ in both microfiber aging and ibuprofen exposure, this result should be interpreted with caution and does not allow the observed difference to be attributed to a single experimental factor.

Polyester aging promotes changes in roughness, surface area, and the availability of functional groups, characteristics that may modify the adsorption of organic compounds [10,16]. Therefore, aged microfibers may interact differently with ibuprofen than virgin

microfibers. However, because adsorption, desorption, and ibuprofen bioavailability in the gastrointestinal tract were not measured in the present study, these processes should be considered plausible hypotheses rather than mechanisms demonstrated by the present results.

Together, these findings demonstrate that different exposure conditions were associated with distinct patterns of HuC/D-IR neuronal density in the intestine of *Danio rerio*. Fish exposed only to polyester microfibers presented lower neuronal density than the control group, whereas groups containing ibuprofen presented higher neuronal density. Although only the comparison between the P and PEIBU groups reached statistical significance, these results reinforce the importance of evaluating emerging contaminants both individually and in combination. Further studies specifically designed to investigate interactions between microfibers and pharmaceuticals, including analyses of adsorption, desorption, and contaminant bioavailability, are needed to clarify the mechanisms underlying these responses.

Conclusions

Exposure to virgin and aged polyester microfibers was associated with lower HuC/D-immunoreactive enteric neuronal density in *Danio rerio* compared with the control group, whereas treatments containing ibuprofen presented higher neuronal density than the control. A significant difference was observed only between the Polyester (P) and Aged Polyester + Ibuprofen (PEIBU) groups. These findings demonstrate that different exposure conditions were associated with distinct patterns of enteric neuronal density in *Danio rerio* and reinforce the importance of evaluating the isolated and combined effects of polyester microfibers and pharmaceuticals to improve our understanding of enteric nervous system responses to mixtures of emerging contaminants.

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