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The Effects of Polyvinyl Chloride Microparticles on the Apoptotic Processes in Zebrafish (*Danio rerio*) Embryos

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Abstract
Pollution caused by plastics is seen as an increasing risk factor for aquatic ecosystems in recent years. Once they are entered to aquatic environment, plastics gradually break down into smaller fragments, and form microparticles (<5 mm in size). These particles are persistent in the environment and cause pollution for a much longer period of time considering their volume and distribution surface. However, the studies about the effects of microplastics on fish are still limited. In whole of the life stages of all metazoan animals, apoptosis is a sine qua non process of tissue homeostasis that requires a sensitive balance between cell renewal and controlled cell death. Although the effects of so many chemicals on apoptosis on zebrafish embryos were widely studied, no report was recorded on the effects of polyvinyl chloride (PVC) microparticles on controlled cell death. For that purpose, zebrafish embryos were exposed 96h to three different concentrations (3, 6, 9 ppm) of PVC microplastics. Acridine orange stain was specifically used to identify the apoptotic cells, and the whole embryos were examined by fluorescein microscopy. Apoptotic cells identified in different parts of embryos were calculated and compared. It was revealed that the effects of PVC microplastics were depended on increased concentrations. As a result, our findings have shown that apoptotic process could be affected by PVC microplastics depending increasing concentrations in zebrafish embryos.

Keywords: PVC microparticle, apoptosis, zebrafish, embryo, acridine orange

1. Introduction

Plastic pollution is the accumulation of any manufactured and persistent plastic objects in the environment. These pollutions adversely affects wildlife, wildlife habitat and humans. (Hammer et al 2012). World production of plastics has strongly expanded during the last decades, from 1.7 million tons in 1950 to 299 million tons in 2013 (Duirs and Coors, 2016). In 2012, 90% of the whole amount of plastic production that reached to 280 million tonnes and these plastics are consisted of; polystyrene (PS) and polyethylene - terephthalate (PET), low-density polyethylene, (LDPE), high-density polyethylene

(HDPE), polypropylene (PP), polyvinyl chloride (PVC). Polyvinyl chloride (PVC) is the one of the oldest and most common plastics. Due to its low cost, durability, and versatility with respect to fabrication and property modifications, PVC is one of the most preferred plastics in use today (Persico et al., 2009). PVC waste leads to the formation of polychlorinated dibenzo-p-dioxins (dioxins) and polychlorinated dibenzofurans (furans), which are both the highly toxic substances (Wagoner 1983).

Plastic particles smaller than 5 mm in size are called as microplastics (MP). These particles proliferate, migrates and accumulates in the natural habitats. These type of pollution is ubiquitous and persistent in the oceans and clearly threatens aquatic habitats (Moore 2008). MPs can enter oceans, lakes and rivers… through solid waste disposal, coastal landfill operations and disposal at sea of solid waste from individual vessels. Major inputs of MPs come from ship generated litter, litter carried to the sea by rivers and the municipal drainage system, and litter left from recreational activities (Cole et al., 2011); but also from fishing fleets (plastic fishing gear), accidently lost, carelessly handled, or land based sources (packing material).

There are two types of plastics can be found on environmental debris. Primary microplastics are type of plastics that have been purposefully created. Other type of them is secondary microplastics which are created from the breakdown of large plastic items.

During the whole life of an organism, tissue homeostasis requires a sensitive balance that is maintained between cell renewal and cell death, and homeostatic cell deletion is controlled via apoptosis. Apoptosis is a form of programmed cell death that plays a pivotal role to maintain homeostasis in all of the metazoan animals (Anvarifar et al., 2016). In contrast to necrosis or traumatic cell death, it is a vital, well-controlled and evolutionary conserved process (Janz et al., 2001).

A wide variety of physiological and pathological stimuli can lead cells to death such as extrinsic stimuli include environmental factors and mechanical injury as well as the actions of infectious organisms or toxic substances; while the intrinsic ones can ben summarized as hormones, orher humoral substances and tissue factors. Generally when the concentration of toxic substance is high enough to cause cellular injury and disorders, cell dies by necrosis through a process without energy consumption. However it is much more common affected cells to die by apoptosis that consumes energy(Anvarifar et al., 2016, Amaral et al. 2007)

Tissue homeostasis can strongly be broken down by MP exposition: exposed, ingested and/or accumulated MPs have the potential to cause a lot of adverse effects such as mortality, reduced feeding activity, inhibited growth and development, endocrine disruption, energy disturbance, oxidative stress, immunity and neurotransmission dysfunction, and even genotoxicity (Lu et al., 2016).

With their highly variable milieu and the unique aspects of biology, fish can offer great advantages for MPs-induced apoptosis. Due to its optical transparency during early development, zebrafish is a very practical model for *in vivo* fluorescence imaging that focused on apoptotic cell death. Stress-induced apoptosis is thought to contribute to abnormal development during embryogenesis (Yabu et al., 2001). In the nervous system and non-neuronal organs of developing zebrafish embryos, apoptosis is also well described (Cole and Rose, 2001, Yamashita, 2003, van Ham et al., 2010). However, no report has been recorded about the relationships between MPs and apoptosis in zebrafish embryos.

2. Material and Method

Fertilized eggs were obtained from mature (7-18 months) male and female zebrafish (*Danio rerio*) in our laboratory. Parents were randomly selected and transferred into two different tanks (28 ±0,5°C temperature, 14h light/10h dark illumination) fed three times a day with commercial fish food (Tetra). PVC microplastics was commercially supplied from Micro Plastic Inc. (Filippines) that produced from larger PVC particles with using a mechanic grinder.

One day before breeding, male and females were placed into breeding chamber with one female/two male ratio. After breeding, collected embryos were washed with E3 media(5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$) then incubated in 28 °C. Within 24 h of spawning embryos were randomly selected and were dechorionized with 0.5 mg/L pronase (Sigma) application, and then transferred into six-well plates. Test groups were exposed to three different microplastic concentration (3, 6, 9 ppm) for 96h. In order to gain a homogenous spreading, exposure chambers were aerated continuously. No chemicals was applied for control group that was also aerated.

After anesthesized with 0,5 mg/L MS222 (Sigma, Cat. No: E10521), embryos were stained with 5 mg/L Acridine Orange (AO) (Sigma, Cat. No: 235474) for 25 min., washed with Phosphate Buffer Saline for three times and examined totally with fluorescein microscope (Leica DM220).

3. Results

In control group slightly stained cells that are on normal developmental apoptotic process were observed (Figure 1 A). When compared to control group, fluorescence expression were increased in all of the experimental groups (Figure 1). In 3 ppm PVC-MPs exposure group along the notochorda, apoptotic accumulation that had not been detected in controls, could be observed easily. Moreover, expression in head region was increased notably (Figure 2). All of the brightened clusters of head and tail regions were significantly increased in 6 ppm PVC-MPs exposition group. Also Brightness observed at last somit of the tail was prominent (Figure 3). In the highest concentration exposition group (9 ppm), the most striking expression was recorded at notochorda, while expression observed in clusters of head region were continued similarly to 6 ppm exposed animals (Figure 4)

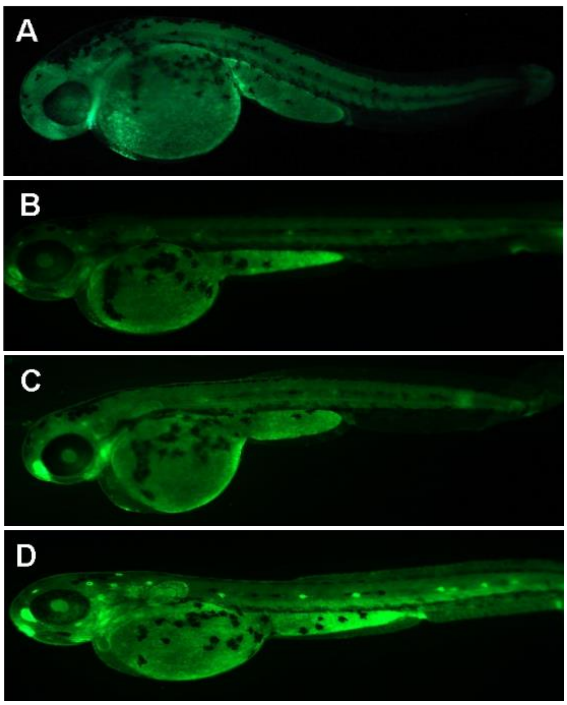

Figure 1. Total body of zebrafish, A: Control Group, B: 3 ppm, C: 6 ppm, D: 9 ppm PVC-MP exposured groups

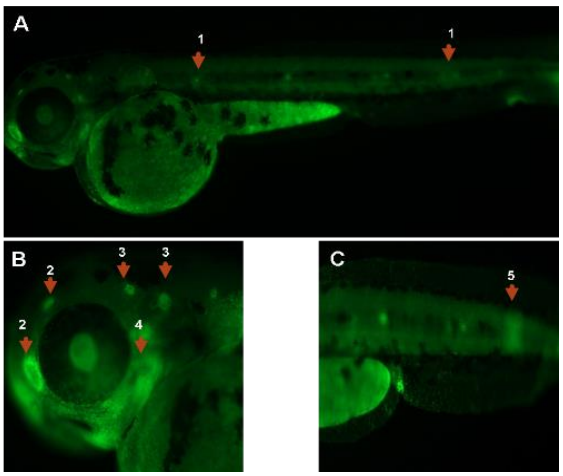

Figure 2: 3 ppm PVC-MP Exporuse group; Along the notochorda, apoptotic accumulation that had not been detected in controls, could be observed easily. Moreover, expression in head region was increased notably (A). Apoptotic cells (A, B and C) Notochord (Arrow 1), Forebrain (Arrow 2), Telencephalon (Arrow 2), Hindbrain (Arrow 3),Around optic capsule (Arrow 4), Tail somits (Arrow 5).

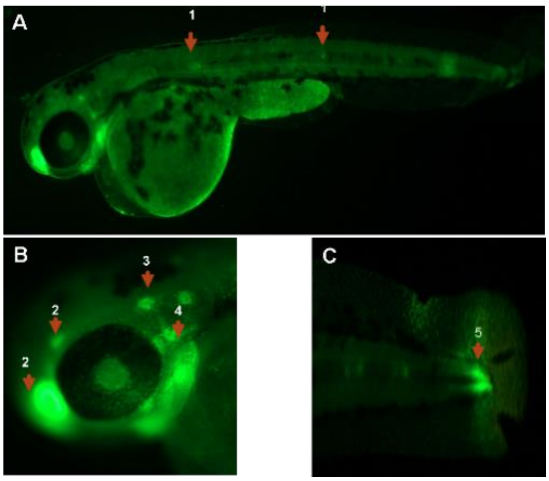

Figure 3: 6 ppm PVC-MP Exporuse group; All of the brightened clusters of head and tail regions were significantly increased. Brightness observed at last somit of the tail was prominent (A). Apoptotic cells(A, B and C): Notochord (Arrow 1), Forebrain (Arrow 2), Telencephalon (Arrow 2), Hindbrain (Arrow 3), Around optic capsule (Arrow 4), Tail somits (Arrow 5).

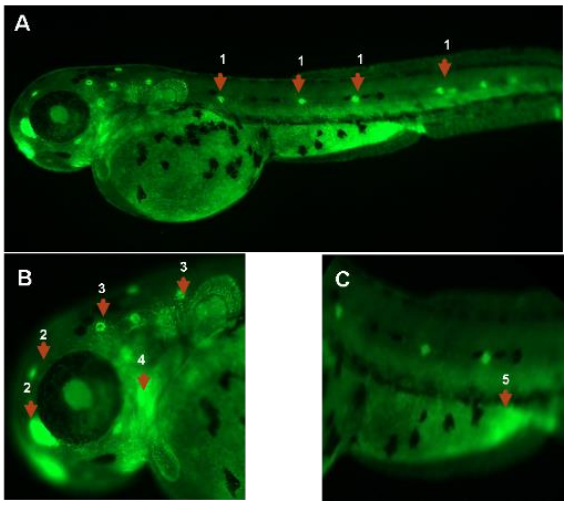

Figure 4: 9 ppm PVC-MP Exporuse; The most striking expression was recorded at notochorda, while expression observed in clusters of head region were continued similarly to 6 ppm exposed animals. Apoptotic cells (A, B and C) Notochord (Arrow 1), Forebrain (Arrow 2), Telencephalon (Arrow 2), Hindbrain (Arrow 3), Around optic capsule (Arrow 4), Tail somits (Arrow 5).

4. Discussion

Zebrafish is the most common laboratory animal for investigating the effects of xenobiotics. Especially in developmental studies, zebrafishes are mainly preferred because of easy to obtain and being transparent embryos and short embryological development. Also it is easy to maintain and adaptation duration in laboratory conditions. Because of all these advantages of zebrafish, investigations that are focused on developmental apoptotic process, zebrafish embryos are mainly used. In this manner, the spatial and temporal patterns of apoptosis during the normal development of the zebrafish embryos were previously described in detail by Cole and Ross (2001).

Apoptotic processes occurred in embryonic developmental stages attract a great deal of attention: rapidly proliferating cell populations have high rates of apoptotic cell death (King and Cidlowski, 1995). During this process, exposure of any kind of toxicant to living organism is induced imbalance between mitosis, so that apoptosis can be triggered and produce developmental abnormalities (Anvarifar et al 2016, Anvarifar et al 2018). For example, cadmium induced oxidative stress in trout hepatocytes result in

apoptosis of damaged cells (Risso-de Faverney et al. 2004). Clearly the relative importance of apoptosis varies with the particular toxicant and tissue.

Here, we were investigated the potential impacts of PVC MPs on developing zebrafish embryos at 6hpf, in the short-term toxicity. In the range of concentrations tested (3, 6 and 9 ppm) MPs of PVC was found to be apoptotic. As we know, this is the first report about the toxicological interactions of MPs of PVC and apoptosis.

In respect to methodology, the most common assay that used for detecting apoptosis is the TUNEL assay, but it can provide false positive signals in some necrotic cells (Ribble et al., 2005): As being a nucleic acid-specific ultraviolet fluorochrome, acridine orange (AO) that stains DNA to bright yellowish-green on a typically green cytoplasmic background, is really a fast and easy method to observe apoptosis with high accuracy.

As expected, apoptotic effects of PVC MPs are related to increasing concentrations. However, we cannot simply conclude that MPs are more effective in embryos than adults. Our preliminary findings can only provide new insights into the toxic effects of MPs on fish. In areas such as sensory organs and the brain transient high rates of cell death have been noted and correlated with morphogenetic and histogenetic process.

It is strongly possible that, the effects are neurotoxic: the adults exposed to PVC MPs in their developmental stage, would be expressed some neurological malformations such as swimming disorders and sight loss (Kabashi et al. 2010)

Despite the increasing numbers of the studies focused to investigate the mechanisms of the toxic effects of MPs are not yet elucidated, and need further study.

MPs can cause neurotoxic disorders either directly or indirectly. They can induce the formation of reactive oxygen species and oxidative stress, cause a decrease in the phagocytic activity of immune cells. Thus, some neurotoxic effects can be triggered indirectly. Dong et al. (2002) were reported that TCDD-induces apoptosis in the midbrain of the zebrafish embryo secondary to local circulation failure, which could be related to oxidative stress. Lu et al. (2016) were also pointed to the role of oxidative stress in zebrafish exposed to polystyrene MPs.

In conclusion there is no doubt that, “sine qua non” properties of apoptosis serve to bring more understanding on the effects of MPs on aquatic environment. As properly noted by Duirs and Coors (2016) the release of plastics in to the environment should be reduced in a broad and global effort regardless of a proof of an environmental risk.

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