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An approach to clarify the effect mechanism of glyphosate on body malformations during embryonic development of zebrafish (*Daino rerio*)

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HIGHLIGHTS

- We try to clarify possible toxic mechanism of Glyphosate on body malformations during embryonic development of zebrafish.
- Glyphosate exposure caused an inhibition effect of carbonic anhydrase enzyme.
- Lack of CA activity lead to increase CO_2 and respiratory acidosis in whole body resulting produce of ROS in gill.
- These increases and abundance of ROS can be considered causing malformations due to the cellular apoptosis.

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ABSTRACT

In this study, it has been investigated that the effects of glyphosate, which is a herbicide within organophosphate and unselective widely used in agriculture on enzyme activity of carbonic anhydrase, production of reactive oxygen species, cell apoptosis and body morphology during the embryonic development of zebrafish.

To this end, it has been treated embryo with 1, 5, 10 and 100 mg/L gyphosate at 96 h. The embryos treated with glyphosate from 4 hpf were evaluated by considering the survival rates, hatching rates, body malformations under the stereo microscope in 24, 48, 72 and 96th hours. In order to clarify the mechanism of the abnormalities ROS, enzyme activity of carbonic anhydrase and cellular death were detected end of the 96th hour.

The data obtained in the present study have shown that glyphosate treatment inhibited CA activity, caused production of ROS especially branchial regions, triggered cellular apoptosis and caused several types of malformations including pericardial edema, yolk sac edema, spinal curvature and body malformation in a dose-dependent manner. As a conclusion, in light of present and previous studies, we can deduce that (1) the probable reason of ROS production was CA inhibition via decreasing of CO_2 extraction and developing respiratory acidosis (however, one needs to clarify), (2) abundance of ROS triggered cellular apoptosis and (3) as a result of cellular apoptosis malformations increased. These data will enable us to further understand potential toxic mechanism of glyphosate on embryonic development stage of zebrafish and may be useful for assessment in the toxicology studies.

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1. Introduction

It is well known that pesticides are chemical substances, biological agent (such as a virus or bacteria), antimicrobial, disinfectant or device used against to pests. Although here are some

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benefits to the use of pesticides, they may also cause various side effects, e.g. modify the structure of DNA (Lee and Steinert, 2003), cause sperm malformations (Mathew et al., 1992), generate reactive oxygen species (ROS) (Bagchi et al., 1995), influence antioxidant defense system (Topal et al., 2015), cause cellular apoptosis (Wu et al., 2015; Yu et al., 2015) and act as inducers of heat shock protein (Ceyhun et al., 2010a) in tissues and cells in different organisms. Especially these toxic contaminants cause cytotoxic effects by the production of a ROS which can induce oxidative damage and perhaps a mechanism of toxicity for aquatic organisms living in polluted areas (Pandey et al., 2003; Li et al., 2010).

Glyphosate (N-(phosphonomethyl) glycine) is used to kill weeds, especially annual broad leaf weeds and grasses known to compete with commercial crops grown around the globe (Ceyhun et al., 2010a). Glyphosate is a broad-spectrum, systemic, post-emergence herbicide that is phloem mobile and is readily translocated throughout the plant (Franz et al., 1997). From the leaf surface, glyphosate molecules are absorbed into the plant cells were they are translocated to meristematic tissues (Laerke, 1995). Glyphosate's primary action is the inhibition of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), a chloroplast-localized enzyme in the shikimic acid pathway of plants (DellaCioppa et al., 1986). This prevents the production of chorismate which is required for the biosynthesis of essential aromatic amino acids. Glyphosate is commonly used for agriculture, horticulture, viticulture and silviculture purposes, as well as garden maintenance (Ceyhun et al., 2010a). However, the use of commercial glyphosate has dramatically increased in recent years (Kreutz et al., 2011). Glyphosate is very toxic to the most organisms including fishes (Folmar et al., 1979). Although there are quite few papers elucidating the toxic effects of glyphosate on organisms (Lopes et al., 2014; Armiliato et al., 2014; Webster et al., 2014; Sandrini et al., 2013), there are still many mechanisms waiting to clarify.

Zebrafish (*Danio rerio*) have long been the animal model of choice for vertebrate developmental studies, as it provides several advantages for investigating organ and tissue development not available through other model systems (Westerfield, 2007). Therefore, it makes logical sense that zebrafish have become a powerful model organism for investigating the molecular and cellular mechanisms by which environmental chemicals disrupt normal developmental processes (Carvan et al., 2005).

Carbonic anhydrases (CAs, E.C.: 4.2.1.1) are ubiquitous zinc containing metalloenzyme family that are responsible for the reversible hydration of carbon dioxide in a reaction $CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^-$. This enzyme family is produced in a variety of tissues where they participate in several biological processes; acid-base balance, respiration, carbon dioxide and ion transport, bone resorption, ureagenesis, gluconeogenesis, lipogenesis and body fluid generation (Hynninen et al., 2004; Kucuk and Gulcin, 2016). Esbaugh and Tufts (2006) reported that the first function of the enzyme is to facilitate the transport of CO_2 into the capillaries by hydrating CO_2 at the capillary wall, where its second function is to help equilibrate the post-capillary pH. Studies shown that some isoenzyme of CA (CA III) functions as an oxyradical scavenger and thus protects cells from oxidative damage (Raisanen et al., 1999).

Reactive oxygen species (ROS), in particular hydroxyl and peroxylradicals, hydrogen peroxide and superoxide radical anion, have long been implicated in oxidative damage inflicted on fatty acids, DNA and proteins as well as other cellular components (Krumova and Cosa, 2016). Although organisms have various antioxidants and detoxifying enzymes to scavenge ROS efficiently, studies have shown that ROS can induce programmed cell death (apoptosis) in many different cell systems (Pierce et al., 1991; Watson et al., 1997; Kasahara et al., 1997; Simon et al., 2000). Apoptosis defined by internucleosomal DNA fragmentation, chromatin condensation, cellular shrinkage and membrane blebbing resulting in the formation of apoptotic bodies (Topal et al., 2014). Programmed cell death occurs normally aging and during development and as a homeostatic mechanism to maintain cell populations in tissues (Susan, 2007). Apoptosis also occurs as a defense mechanism such as in immune reactions or when cells are damaged by disease or xenobiotics (Norbury and Hickson, 2001; Susan, 2007).

In this study, the actual toxic mechanism of glyphosate treatment on body malformations result from cellular apoptosis caused by the CA inhibition and/or ROS generation has been investigated during embryologic development in zebrafish (*Danio rerio*) which is an appropriate model organism for toxicological experiments.

2. Materials and methods

2.1. Zebrafish maintenance and embryo treatment

AB strain zebrafish (*Danio rerio*) were obtained from Oregon State University (US) and were kept in Aquatic Habitats (Imported by Akuamaks Co., Turkey) zebrafish system which was maintained a constant temperature of 28 °C under a 14:10 h light-dark photoperiod. The fish were fed with *Artemia salina* twice a day. Zebrafish embryos were obtained from spawning adults in groups of about 20 males and 10 females in tanks overnight. Spawning was induced in the morning when the light was turned on. Embryos were examined at 4 hpf (hours post fertilization) under a dissecting microscope, and unfertilized and death embryos were removed. Selected 40 embryos for each group, that had developed normally and reached the blastula stage, were treated graded concentrations of dosing solutions (1, 5, 10, and 100 mg/L) of glyphosate which were prepared in E3 embryo medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, %0,01 methylene blue) (Westerfield, 2007). The commercial formulation of glyphosate (N-phosphono methylglycine, 360 mg/L) was used. Mediums renewed every 24 h. The concentrations were selected based on our previous studies (Topal et al., 2015), and information from the available literature (Webster et al., 2014; Lopes et al., 2014). Three replicates for each concentration were used. Mortality was identified by coagulation of the embryos, missing heartbeat, failure to develop somites and a non-detached tail (Shi et al., 2008). Dead embryos were recorded and promptly removed from the solution during observations. During the 96 h of exposure, embryos and larvae were examined under a stereomicroscope (Zeiss, Discovery V12, Germany) to screen for morphological abnormalities (included pericardial edema, yolk sac edema, body malformation and spinal curvature) and recorded at 24, 48, 72 and 96 hpf among the embryos and larvae from both the control and treated groups. 3% Methyl cellulose was used for immobilized larvae during imaging.

2.2. CA enzyme activity assay

For enzyme activity end of the treatment (at 96 h) randomly selected 10 larvae from each group (control, 1, 5, 10, and 100 mg/L) were homogenized via Tissue Laser (Qiagene) in appropriate amount Tris-HCl buffer, then centrifuged at 10,000*g* for 30 min at 4 °C, supernatant was used for activity determination. CA enzyme activity was assayed by following the hydration of CO_2 according to the method described by Wilbur and Anderson (1948). CO_2-hydratase activity as an enzyme unit (EU) was calculated by using the equation $(t_0\text{-}t_c/t_c)$ where t_0 and t_c are the times for pH change of the nonenzymatic and the enzymatic reactions, respectively (Alım et al., 2015; Huyut et al., 2016).

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