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Preliminary communication

ASSESSMENT OF SUBACUTE GENOTOXIC AND HISTOPATHOLOGICAL EFFECTS OF A FOOD FLAVOUR INGREDIENT, 4-ETHYLBENZALDEHYDE (EBA) ON ZEBRAFISH (*DANIO RERIO*) MODEL

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Modern food industry widely uses a variety of flavour and fragrance materials. One of the most used compound groups is the aldehydes. The benzaldehyde, also known as artificial almond oil, is one of the most commonly used flavouring in food industry nowadays. The effects of this compound on different species are well known, a lot of toxicological information can be found in the literature. 4-ethylbenzaldehyde is also a member of aldehyde group, the physical properties are similar to benzaldehyde and also has almond scent. Unlike benzaldehyde, it has no chemical safety assessment according to its chemical safety sheet, and only one experiment can be found on its effects on vertebrates. This compound can also be found at the group of flavours and fragrances. The aim of this study was to examine the subacute DNA and tissue damaging effects of EBA. The genotoxic effects of EBA in zebrafish were evaluated by using micronucleus assay. Significant increase in the micronucleus frequency had been described for all tested concentrations. Alterations were found in the liver of the fish group treated with 11 mg l^{-1} EBA for 21 days.

Keywords: 4-ethylbenzaldehyde, flavours, fragrances, genotoxicity, micronucleus assay, zebrafish

Modern food industry widely uses a variety of flavours and fragrances. Among these numerous compounds are of natural origin, but some of them are synthetically produced. Many of these substances have been proven to be toxic, although these compounds are still used in the industry or were used until they were banned. For example, various sweetening agents and artificial dyes (Weihrauch & Diehl, 2004; Huff & Ladou, 2007; Amchova et al., 2015) as additives play important roles in modern food processing, an average consumer take them in every day, it is a high-priority to know its risks for human health.

Food industry uses the group of aldehydes for a long time. The benzaldehyde, also known as artificial almond oil, is the second most commonly used artificial aroma after artificial vanilla (Krings & Berger, 1998). Benzaldehyde has comprehensive toxicological literature (American College of Toxicology, 2006), its chemical safety sheet contains a considerable amount of useful data (CAS 100-52-7). Acute results are very important, but

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people might get in contact with this material every day, like with EBA, so great attention should also be given to the chronic and subacute effects. It is less toxic to mammals (rabbit, rat, mouse, guinea pig), acute oral LD_{50} values are really high (higher than 1000 mg kg^{-1}), except mice (oral LD_{50} 28 mg kg^{-1}). Abramovici and Rachmuth-Roizman (1983) had studied the effects of benzaldehyde on chicken embryos, they have found an increasing number of abnormal development of skeleton and limbs in a concentration dependent manner. According to the WHO (1996), the benzaldehyde causes delayed development, decreased fetal and neonatal weights in mice, rats, hamsters, and rabbits, but only in concentrations proved to be toxic to the parents. Acceptable daily intake of benzaldehyde is 5 mg kg^{-1} body weight and estimated daily intake of benzaldehyde is 9300 µg/capita/day in Europe and 36 000 µg/capita/day in the USA (WHO, 2002).

The compound (4-ethylbenzaldehyde) belongs to the group of aldehydes. Its physical properties are identical to benzaldehyde. It is a colourless, almond scented liquid. It can be found during analysis of fragrance components of different kind of foods, for example in lettuce and cabbage (Lonchamp et al., 2009), in French beans (Barra et al., 2007), and also in green tea (Shimoda et al., 1995). It has also been described as a scent component of foodstuffs of animal origin, for instance in matured anchovy (Triqui & Reineccius, 1995), in mussels (Le Guen et al., 2000), and in different kind of sea-fish (Morita et al., 2003; Silva et al., 2012). Less known is the fact that EBA is also known as a water disinfection by-product (Rácz et al., 2012). The study of this compound is very important for the preservation of human health, because we can get in contact with it in many areas of life.

Unlike benzaldehyde, according to the safety data sheet of EBA (CAS 4745-78-1), it has no chemical safety assessment or toxicity data. The compound is included in the scientific opinion on flavouring group evaluation issued by the European Food Safety Authority (EFSA) (2012). In this EFSA publication, there is no security concern with EBA, it has been classified as flavouring agent for foodstuffs. There is only one publication mentioning this compound in scientific sense, an unpublished report from 1984, but the MSDI value of EBA had been determined (0.37 µg/capita/day) (EFSA, 2012). In contrast, according to the WHO (2002), threshold for human intake for the structural class of EBA is 1800 µg/day, and there is no safety concern at current level of intake when used as a flavouring agent. In Regulation (EC) No 1272/2008 of the European Parliament and Council, it is not considered dangerous substance (EC, 2008). The currently available limited information on EBA is worrying. Up to now, it has only been published in one study on vertebrates. In that study (Rácz et al., 2012), the subacute effects of the compound have been studied on zebrafish model. The main goal of this paper is to increase knowledge on the effects of EBA on living organisms, thus contributing to consumer's safety.

1. Materials and methods

1.1. Experimental substance

Stock solution of 4-ethylbenzaldehyde (EBA) (purchased from Sigma Aldrich Hungary, CAS 4745-78-1) has been prepared in distilled water, by using ultrasonication (amplitude: 20%, time: 4 min, Branson Digital Sonifier 250, Branson Ultrasonics Corp., USA). Solutions for treatments have been prepared with the water of ZebTEC (Techniplast S.p.a.) fish maintenance system.

1.2. Treatment

This experiment has been made on laboratory cultured 'AB' zebrafish line, in compliance with the applicable animal welfare regulations. Before the experiment, fish had been kept in Techniplast ZebTEC laboratory recirculation system (at 27 °C water temperature, with 14 h light and 10 h dark periods, pH 7.0±0.2, conductivity: 525±50 μS). Fish were fed twice a day with complete SDS Small Gran dry fish food (Dietex International Limited Special Diets Services G.B.). This feeding method was used during the treatment. The treatment was done in semi-static system, test solutions were changed every second day. The experiment has lasted 21 days. Concentrations were defined according to the results of RÁCZ and co-workers (2012). The highest applied concentration was the LC_{10} value (calculated concentration of compound at which 10% of treated fish is expected to die) of that study, calculated by authors of the cited article as 11 mg l^{-1} to avoid dying of the test animals and to induce sublethal symptoms. Also, 5.5 and 2.75 mg l^{-1} concentrations were examined. In case of control groups only the water of fish keeping system has been used. Adult (approximately 6–8 months) male and female fish were also used during the experiment in 3 groups/dose level with 8 fish/group.

1.3. Micronucleus assay

Induction of micronuclei (MNi) formation has been determined in erythrocytes isolated from fish exposed to 4-ethylbenzaldehyde for 1, 2, and 3 weeks (4 fish per concentration). After obtaining peripheral blood samples, they were immediately smeared on microscope slides (Menzel-Gläser). Slides were left to air-dry and then stained with Hoechst 33342 dye (5 μM). The stained slides were examined under an epifluorescence microscope (Olympus BX-51, Tokyo, Japan) at a magnification of ×400, and evaluated for the presence of MNi exhibiting blue fluorescence in the peripheral blood erythrocytes. A total of 2000 randomly selected cells with complete cytoplasm were examined from each slide. The criteria for the identification of fish micronucleated erythrocytes were as follows: (a) MNi should be smaller than one-third of the main nuclei, (b) they should be on the same plane of focus, (c) MNi must be of the same colour and intensity as the main nuclei, (d) they should have oval or round shape, and (e) they should be clearly separated from the main nucleus.

1.4. Statistics

Data were analysed by using descriptive statistics and the results are presented as mean±SD. Statistical evaluation of the micronucleus assay data was performed with STATISTICA 12 software package (StatSoft, Tulsa, OK, USA). For the data from the micronucleus assay, Poisson regression was used to compare with controls and varying concentrations of 4-ethylbenzaldehyde. $P<0.05$ was considered significant.

1.5. Histopathology

Zebrafish were fixed in 4% buffered formaldehyde for 24–48 h at 4 °C with opened abdominal cavity, washed with phosphate buffered saline (PBS), and tissues were dehydrated in a series of graded ethanol solutions and xylene before embedment in paraffin. Fish were placed into the cassette for sectioning. Sections were 4–6 μm thick and were stained with hematoxylin and eosin (HE). Two males and two females had been used from both treatment concentrations.

2. Results and discussion

2.1. Micronucleus assay

The genotoxic effects of 4-ethylbenzaldehyde in zebrafish were evaluated by using micronucleus assay. The main goal of the 21-day experiment was to examine subacute chromosome damaging activity of the compound.

Poisson regression showed significant increase in the micronucleus frequency for all concentrations tested, compared with control groups. Increasing number of MNi were found after the first week, compared to the control group (1.00±0.82), even in the lowest applied concentration (2.00±0.82). Significant increase was noticed only after 3 weeks exposure for all concentrations tested. In case of 2.75 mg l^{-1} the number of MNi formed was 2.75±2.22, for 5.5 mg l^{-1} and 11 mg l^{-1} treatments the numbers of formed MNi were 3.50±1.29 and 3.50±1.00, respectively, at the end of the experiment (Fig. 1, Table 1). No statistically significant differences were observed among treated groups during the experiment in case of applied concentrations, so MNi formation was not concentration-dependent. Applied concentration range was probably not sufficiently wide to detect concentration-dependent changes.

This method has been widely applied for a long time (Almássy et al., 1987; Nersesyan et al., 2014; Shimada et al., 2015). According to the results of this study, it is suitable to detect the genotoxic effects of EBA in low concentrations.

Similarly to acute studies, due to its structural and physico-chemical similarity, it is assumed that the chronic effects of EBA may be similar to benzaldehyde.

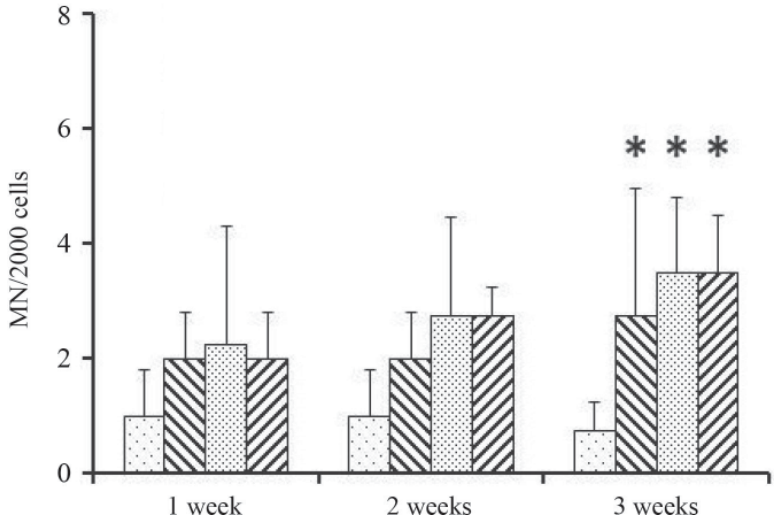

Fig. 1. 4-Ethylbenzaldehyde-induced micronuclei (MN) in zebrafish erythrocytes. Zebrafish were exposed to different concentrations of 4-ethylbenzaldehyde (0, 2.75, 5.5, and 11 mg l^{-1}). The micronuclei frequency was determined in the F1 zebrafish treated with 4-ethylbenzaldehyde for 1, 2, and 3 weeks. Data are presented as mean±SD from four individuals. * Statistically significant difference compared to corresponding control ($P<0.05$)
⚀: 0 mg l^{-1}; ⚁: 2.75 mg l^{-1}; ⚂: 5.5 mg l^{-1}; ⚃: 11 mg l^{-1}

In case of chronic exposure, benzaldehyde has teratogenic (Abramovici & Rachmuth-Roizman, 1983), mutagenic (Haworth et al., 1983), and carcinogenic (NTP, 1990) effects. In spite of all these, US Food and Drug Administration (US FDA) has generally recognised it as safe (GRAS). EFSA also assessed the potential carcinogenicity of EBA and indicated that the compound is not carcinogenic and non-genotoxic (EFSA, 2012).

Table 1. 4-Ethylbenzaldehyde-induced genome damage. The frequency of 4-ethylbenzaldehyde-induced micronuclei (MNi) in zebrafish erythrocytes was assessed with the MN assay

EBA (mg l^{-1})	Sample No.	MNi/2000 cells		
		1 week	2 weeks	3 weeks
0 mg l^{-1}	1	0	0	1
	2	2	1	1
	3	1	2	0
	4	1	1	1
	Σ	1.00±0.82	1.00±0.82	0.75±0.50
2.75 mg l^{-1}	1	3	2	6
	2	1	2	2
	3	2	3	2
	4	2	1	1
	Σ	2.00±0.82	2.00±0.82	2.75±2.22*
5.5 mg l^{-1}	1	1	1	3
	2	4	5	5
	3	0	2	4
	4	4	3	2
	Σ	2.25±2.06	2.75±1.71	3.50±1.29*
11 mg l^{-1}	1	3	2	3
	2	2	3	3
	3	2	3	3
	4	1	3	5
	Σ	2.00±0.82	2.75±0.50	3.50±1.00*

Zebrafish were exposed to 4-ethylbenzaldehyde (0, 2.75, 5.5, and 11 mg l^{-1}) for 1, 2, and 3 weeks as described in Materials and methods. Data are presented as mean±SD number of MNi/2000 from four individuals. Significant difference between treated fish and the corresponding control is indicated by asterisk (*) ($P<0.05$).

2.2. Results of histopathology

Alterations were found in the liver of the group of fish treated with 11 mg l^{-1} EBA for 21 days. Within the liver parenchyma cells, changes were observed in the distribution and relative content of fat. Fat droplets nearly filled the whole cytoplasm and varied in size (Fig. 2). These slight lesions were observed in both sexes. No serious lesions, like adenofibrosis or hepatocyte megalocytosis were found. Rácz and co-workers (2012) applied three months treatment time and lower concentrations (2.5 mg l^{-1} and 5 mg l^{-1}). Symptoms observed in the recent study are similar to their 5 mg l^{-1} results. According to these, the substance in a shorter treatment time

with higher treatment concentration also has significant effect on the liver, the main detoxifying organ. In case of other organs (gill, kidney), considerable lesions were not experienced. Presumably to the development of these lesions takes more time, because RÁCZ and co-workers (2012) described lesions in the mentioned organs.

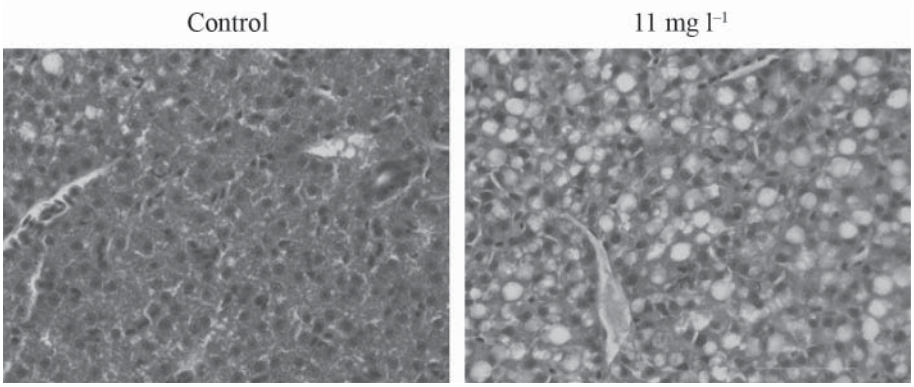

Fig. 2. Histological sections of control and treated fish. Images showing liver of a control fish with moderate fatty change (left) and severe diffuse fatty change in the liver of a fish treated for 21 days with EBA (right). (H-E ×400)

3. Conclusions

Based on our results, 4-ethylbenzaldehyde has toxic effect at the tested concentrations. In case of micronucleus assay, EBA caused significant increase in MNi formation in treated groups compared to control group, but it was not concentration-dependent. We can conclude that EBA has DNA damaging effect at lower concentrations and applied concentration range was not wide enough to detect concentration-dependent effects. At the highest applied concentration (11 mg l^{-1}) it caused fat infiltration in liver after 21 days of treatment. Applying longer treatment time may cause alterations in other organs.

In the future, it would be useful to examine a wider range of concentrations with this method and also to apply other genotoxicity tests, for example Comet Assay, on adult fish and embryos. It would be useful to examine the effects of this compound with molecular toxicological methods, for instance microarray assay.

Examined concentrations in this study are higher than EFSA limit, but there are difficulties in determination of real human and environmental expositions. It may also occur in some foods naturally or as an additive in cosmetics and in drinking water. Our results have shown that it is important to examine compounds recognised as safe, because they may have hidden dangers.

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