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Toxic effects of acrylamide on survival, development and haemocytes of *Musca domestica*

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ABSTRACT

The influence of acrylamide, a potentially toxic substance present in some types of food, on survival, postembryonic development and haemocytes, insect's blood cells, of the housefly was examined. Larvae were reared on media contaminated with acrylamide at concentrations of 82 μg/g, 164 μg/g or 246 μg/g. The length of larval and pupal stages as well as the survival of larvae and pupae was examined. To study the effects of acrylamide on haemocytes, the analysis of their index and morphology was performed in the third instar larva. The obtained data showed that the survival of larvae exposed to 82 μg/g and 164 μg/g concentrations of acrylamide decreased by 50% and 85%, respectively, whereas 246 μg/g concentration was lethal. In both groups of flies, larval and pupal stages were significantly lengthened by about 1.5 day in comparison with control. Moreover, acrylamide increased the number of prohaemocytes and intermediate cells while the number of plasmatocytes and granulocytes decreased. The size of plasmatocytes decreased in acrylamide-treated larvae when compared with these cells of control flies. The reduced survival of animals is probably due to affecting haemocytes involved in immune responses in insects. Moreover, the housefly's blood cells showed to be sensitive to toxin, which suggests their usefulness to test toxicity of substances present in food products.

1. Introduction

Acrylamide ($H_2C{=}CH{-}CO{-}NH_2$) is a highly reactive molecule mainly used for polyacrylamide synthesis. Polymeric form of acrylamide has many applications as a soil conditioner, cosmetic stabilizer and as an ingredient in paper and in textile productions. In laboratory, acrylamide is used in electrophoretic techniques (Friedman, 2003). Polyacrylamide is not a toxic agent whereas its monomer has been reported to be neurotoxic, genotoxic and carcinogenic in rodents (Park et al., 2002; Lehning et al., 1998).

The worldwide concern about acrylamide toxicity began in 2002 when the Swedish National Food Administration announced that high concentrations of acrylamide are present in some food products. Acrylamide is a product of the Maillard's reaction during starch food processing at high temperatures, and the yield of this reaction depends on temperature and amounts of carbohydrates and amino acids in food (Mottram et al., 2002; Stadler et al., 2002).

The Maillard reaction, responsible for food taste and its brown colour, utilizes reducing sugars and free amino groups leading to glycosilamine formation and then Amadori rearrangement products. After degradation, highly reactive compounds like furfural, reductones, acetol or pyruvaldehydes condense with free amino groups forming aldehydes and α-aminoketones. Finally, a set of reactions leads to melanoidins, brown nitrogenous polymers (Martins et al., 2001; Taeymans et al., 2004; Zhang and Zhang, 2007). Mottram et al. (2002) have established that $-NH_2$ groups of asparagine and methionine in a presence of dicarbonyls from the Maillard reaction are the main substrates for acrylamide synthesis. However, Zyzak et al. (2003) have revealed that rather carbonyls are required for acrylamide formation from asparagine instead of dicarbonyls. Binding carbonyl source to asparagine forms Schiff base which after decarboxylation process leads to acrylamide and imine molecules. It has also been shown that the presence of enzymes from the group of decarboxylases and temperature could easily degrade asparagine, forming acrylamide intermediate, 3-aminopropionamide (Zyzak et al., 2003) directly leading to acrylamide synthesis in carbohydrates-free environment. It confirmed the previous findings of Stadler et al. (2002) that the carbon skeleton of acrylamide molecule derives from asparagines. Stadler et al. (2002) have also revealed that water significantly increases acrylamide concentration to almost three folds of its content found in anhydrous conditions. Temperature elevation to 170 °C leads further to an increase of acrylamide production.

High content of carbohydrates and asparagine, e.g. in potatoes, wheat and rye grains (cereals), almonds and coffee beans enhances

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acrylamide formation during frying or baking processes (Tareke et al., 2002; Zhang and Zhang, 2007). The concentration of acrylamide noted during thermal processing of this foodstuff reaches the level of 3 μg/g in French fries and even 7 μg/g in roasting coffee beans. It must be noted, however, that not only pH, processing temperature and moisture influence acrylamide formation but also plant cultivating, food storage, reducing sugar content in prefabricates and processing time (Zhang and Zhang, 2007).

Acrylamide may be absorbed through skin, alimentary tract and respiratory system and spread in the body with blood and other fluids (Calleman, 1996; Friedman, 2003; LoPachin, 2004). It also passes the blood-brain and placental barriers (Sörgel et al., 2002; Schettgen et al., 2004). The metabolism of acrylamide is complex and not fully understood yet. Friedman (2003) and our earlier studies have shown that the double bond of acrylamide reacts especially with –SH groups of cysteine/cystine and $–NH_2$ groups of lysine resulting in disruption of cell homeostasis. In red blood cells the toxin forms adducts with haemoglobin or other intracellular proteins. The likely pathway of acrylamide detoxification is active conjugation with glutathione and passive adduction to haemoglobin and serum albumins in blood (Tong et al., 2004; Paulsson et al., 2005). In the housefly, we have found that the addition of proteins rich in sulfhydryl groups to the larval medium decreases toxic effects of acrylamide (Banach et al., unpublished results).

The housefly is often used as a model species to test insecticides but has never been used to test a potential toxicity of substances which are present in food products. These substances originate from contaminations in the environments, food processing and preservation and could be dangerous for any organism, including humans. It has been found however, that some chemicals e.g. heavy metals which are toxic for humans are also toxic for flies and other insects affecting their survival, development and immune responses (Borowska and Pyza, 2004). The immune responses depend, among others, on haemocytes present in haemolymph (insect's blood), which participate in the removal of bacteria and other pathogens from the body.

The aim of the present study was to examine effects of acrylamide on the survival of larvae and pupae of the housefly, its development and on the number and morphology of different classes of haemocytes present in the housefly's haemolymph.

2. Materials and methods

2.1. Animals

The housefly *Musca domestica* was kept in 0.03 m^3 cages at 24 °C, in a light/dark cycle LD12:12 (12 h of light and 12 h of darkness) and fed with a mixture of milk powder, sugar and water. Flies laid eggs on a surface of the medium containing standard rabbit pellets (200 mg) and milk powder (10 mg) mixed thoroughly with water (400 ml). Then, 300 mg of eggs (about 3000 eggs) was transferred to the fresh medium containing rabbit pellets, milk powder and water mixed with 50 mg, 100 mg or 150 mg of acrylamide powder (acrylic acid amide, C3H5NO, ⩾99%, EU# 201-173-7, CAS# 79-06-1, A 3553, Sigma–Aldrich Chemie GmbH) or on the control medium. After pupation insects were transferred into cages and adult flies were no longer exposed to acrylamide.

The final concentration of acrylamide in the rearing media for larvae was 82 μg/g (ACR_{82}), 164 μg/g (ACR_{164}) or 246 μg/g (ACR_{246}), respectively. We used high concentrations of acrylamide since our preliminary study on the survival of the housefly's and the fruit fly's (*Drosophila melanogaster*) larvae has shown that acrylamide concentration equal to 82 μg/g (ACR_{82}) is the mean lethal concentration (LC_{50}) for both species. So, the maximum concentration of acrylamide for a larva per day was 2.2 μg as calculated on the basis of mean hatching efficiency of animals kept in control conditions (76%), and the mean body mass of the third instar larva after 10 days of development (24 mg). Thus, the 82 μg/g of acrylamide as the mean lethal concentration for the housefly seems to be appropriate to observe behavioural, physiological and cellular responses to its toxicity. Moreover, this concentration revealed clear responses of the animal model tested and was similar to that used by Barber and LoPachin (2004) and LoPachin et al. (2004) who treated rats with 50 μg/g per day and 21 μg/g per day for acute and chronic experiments ad libitum and intraperitoneally, to obtain detectable effects of this substance on the nervous system.

2.2. Animal behaviour and survival

Behaviour of larvae, kept on different experimental media contaminated with acrylamide, was observed during larval development to establish their direct response on acrylamide in rearing medium. Larval and pupal rate of development was examined in all experimental groups and compared with control. The influence of acrylamide on the larval survival was calculated on the basis of the number of larvae which hatched from 300 mg of eggs and were able to pupate. The pupal survival was calculated as the ratio of the number of pupae and the number of adults emerged from the pupae.

2.3. Analysis of haemocyte index and haemocytes morphology

Third instar larvae were rinsed with water and placed on ice for immobilization. Next, 10 larvae were cut laterally at the anterior part of the body with a microdissecting scissors and 30 μl of haemolymph was collected using calibrated microcapillaries and diluted in 300 μl of Hanks' Balanced Salt Solution (HBSS) with addition of 1 mM ethylenediaminetetraacetic acid (EDTA). The haemolymph solution was centrifuged for 10 min at 800*g* at 21 °C (1K15, Sigma, Germany), the supernatant was removed and cell pellet diluted again with 300 μl of HBSS with EDTA. Then, the cell solution was centrifuged for 10 min at 200*g* using a cytospin centrifuge (Rotofix32, Hettich, Germany), and the haemocytes were finally fixed with a cold solution of acetone and methanol (1:1) for 5 min. Fixed samples were stained with May-Grünwald dye (Aqua-Med, Poland) for 3 min, washed with a distilled water and next stained with Giemsa (Aqua-Med, Poland) for 15 min. After washing and air-drying the samples were mounted with Permount (Fisher Scientific Company, New Jersey, USA) and analysed under a Nikon Optiphot light microscope (LM) with 100× oil immersion objective and equipped with a digital camera Nikon DXM1200F. The haemocyte classes were determined according Kerkut and Gilbert (1985) and Borowska and Pyza (2004). The following four classes of haemocytes, as in the previous study (Borowska and Pyza, 2004), were identified in the housefly's haemolymph: prohaemocytes (PR), plasmatocytes (PL), granulocytes (GR) and intermediate cells (I). The percentage of each cell type to the total number of haemocytes (the haemocyte index) and all type cell sizes were calculated from randomly chosen 100 images of haemocytes grabbed with a digital camera under light microscope. The areas of haemocytes were measured using image analysis software (Image J v. 1.30z).

2.4. Statistics

The statistically significant differences in the rate of larval and pupal development, the survival of larvae and pupae as well as sizes of four classes of haemocytes between the control and the experimental groups treated with different concentrations of acrylamide were detected using the ANOVA followed by Duncan's test at $p = 0.05$. Changes in percentage of four types of haemocytes in the haemolymph of larvae treated with acrylamide and in control were compared after Bliss transformation of data followed by Kruskal–Wallis H test at $p = 0.01$.

3. Results

3.1. Effects of acrylamide on survival and development

The larvae reared on the media with 82 μg/g, 164 μg/g or 246 μg/g concentration of acrylamide were highly active in all experimental groups on the first day of treatment, penetrating and spreading over the media. After two days the medium with 246 μg/g (ACR_{246}) of acrylamide was found to be lethal for the larvae. Those kept on media contaminated with 82 μg/g (ACR_{82}) (LC_{50}) or 164 μg/g (ACR_{164}) of acrylamide migrated from the rearing medium to its surface or aggregated into small groups, probably trying to avoid contact with the medium.

The survival of larvae reared on the medium with 82 μg/g concentration of acrylamide was decreased by 50%, but in the medium with 164 μg/g of acrylamide only 15% of larvae survived in comparison with control (Fig. 1). The survival of pupae was not changed, however, in both experimental groups and in control when compared with the number of larvae entering the pupal stage in each group (Fig. 1). In the control, larvae pupated synchronously during 1–3 days, whereas in acrylamide-treated groups larval and pupal development was longer and desynchronised between individuals. The length of larval (three larval instars) and pupal stages showed statistically significant increase when compared with the control (Table 1). The larval stage of both ACR_{82} and ACR_{164} groups length-

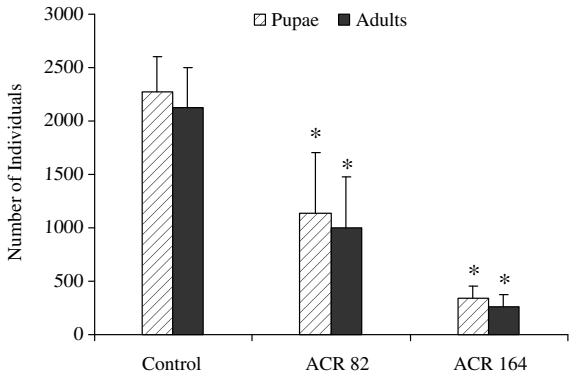

Fig. 1. Survival of larvae and pupae in control medium and after its exposure to different concentrations of acrylamide. The highest concentration of acrylamide used in the present study is not shown due to its lethal effect. * indicates statistically significant differences between contaminated media and control group of animals at $p = 0.05$.

Table 1
Duration of larval and pupal stages in insects reared on the control medium and after exposure to different concentrations of acrylamide: 82 μg/g (ACR_{82}) or 164 μg/g (ACR_{164})

	Larval stage (days)	Pupal stage (days)
Control	9 ± 1	5 ± 1
ACR_{82}	10.5 ± 0.9*	6.8 ± 1.2*
ACR_{164}	10.7 ± 0.6*	5.8 ± 0.8

The highest concentration of acrylamide is not shown here due to its lethal effect. * indicates statistically significant differences between contaminated media and control group of animals at $p = 0.05$.

ened significantly by 1.5 day. The pupal developmental stage, however, was significantly lengthened only in the ACR_{82} group (LC_{50}), whereas the ACR_{164} group showed no difference in the length of pupal stage in comparison with control.

3.2. Haemocyte index and cell sizes

The haemolymph of the housefly's larvae reared on control media contained 48% of prohaemocytes, 20% of plasmatocytes, 24% of granulocytes and 9% of intermediate cells (Fig. 2). The treatment of larvae with acrylamide caused the statistically significant increase of the number of prohaemocytes and intermediate cells,

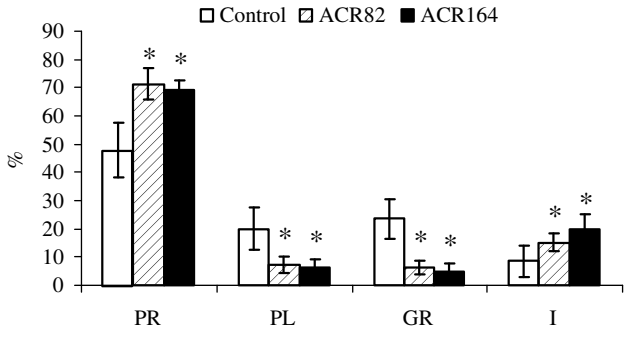

Fig. 2. The percentage of four types of haemocytes identified in the haemolymph of *Musca domestica* larvae on the basis of May-Grünwald–Giemsa staining. The larvae were reared on the media with 82 μg/g (ACR_{82}) or 164 μg/g (ACR_{164}) of acrylamide as well as on the uncontaminated medium as control; prohaemocytes-PR, plasmatocytes-PL, granulocytes-GR, intermediate cells-I. * indicates statistically significant differences between the contaminated media and control group of animals at $p = 0.01$.

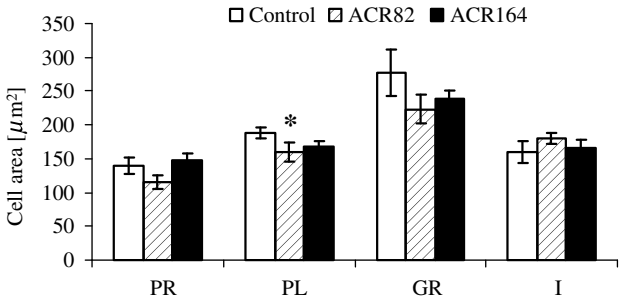

Fig. 3. The cell area (in μm^2) of four types of haemocytes identified in *Musca domestica* larvae treated with two concentrations of acrylamide in the rearing medium: 82 μg/g (ACR_{82}) or 164 μg/g (ACR_{164}) and reared on the uncontaminated medium as control; prohaemocytes-PR, plasmatocytes-PL, granulocytes-GR, intermediate cells-I. * indicates statistically significant differences between contaminated media and control group of animals at $p = 0.01$.

while plasmatocyte and granulocyte numbers were decreased when compared with control.

The measurements of cell size in four classes of heamocytes revealed that the exposure to the low concentration of acrylamide (82 μg/g) influenced their dimensions. The statistically significant decrease of cell size was observed only in case of plasmatocytes, however (Fig. 3).

4. Discussion

The toxic effects of acrylamide, its absorption and metabolism have not been studied in invertebrates. Our earlier study showed that acrylamide affects cell homeostasis in the housefly (Banach et al., unpublished results). This result is based on changes detected in elemental composition of gut epithelial cells of adult flies which were exposed as larvae to acrylamide. To the body of the housefly's larvae, acrylamide is probably absorbed through the digestive system, because the epithelial cells of gut, which showed significant changes in concentrations of Na, Mg, P, S and K have a direct contact with toxins present in food. In addition, there are no barriers protecting these cells against acrylamide, and it seems to be easily transported to all tissues in the body with haemolymph. Thus haemocytes, cells present in haemolymph, are a good target to study toxic effects of acrylamide on cells and on organism condition since these cells play important roles in immune responses of insects to pathogens (Kraaijeveld et al., 2001).

The obtained data showed that survival and development of larvae and pupae exposed to acrylamide are significantly reduced, while the high concentration of acrylamide in the rearing medium (246 μg/g) is lethal for larvae. The larvae, however, posses efficient mechanisms for toxin elimination or inactivation because the ratio of larvae and pupae found in contaminated media was comparable with control. The delay of mitosis might be responsible for significantly longer development of the housefly's larvae exposed to 82 μg/g and 164 μg/g concentrations of acrylamide. In mice it has been found that bone marrow cells of males exposed to 120 μg/g of acrylamide show a delay in cell cycle (Gassner and Adler, 1996). The process of cell division was blocked at metaphase probably due to interactions of the toxin with microtubule associated proteins (MAPs). Titenko-Holland et al. (1998), analysing mouse embryos, have also reported a clear decrease of the number of mitotic figures and increase of mitosis duration. Moreover, the development of larvae and metamorphosis during pupal stage may also be lengthened because of detoxification of acrylamide.

The significant decrease of granulocyte and plasmatocyte numbers with the concomitant increase of prohaemocytes and intermediate cell numbers observed in the present study might be an

effect of reduced differentiation and/or death of granulocytes and plasmatocytes. Due to the lack of granulocytes and plasmatocytes in haemolymph, the resistance of flies to pathogens is decreased, which in turn increases their mortality. A dramatic lost of granulocytes and plasmatocytes might also be a consequence of their higher sensitivity to acrylamide in comparison with prohaemocytes and intermediate cells. Similarly, the number of human lymphocytes decreases after their exposure to acrylamide in concentration of 0.5 μM (Blasiak et al., 2004). Since prohaemocytes and intermediate cells are not differentiated type of cells, the influence of toxin on their metabolism and especially on cytoskeleton may be diminished. The similar effect was observed in undifferentiated neuroblastoma SH-SY5Y cells that have been found to be more resistant to acrylamide than differentiated cells of the same cell line (Hartley et al., 1997), probably due to insufficient development of neurofilaments.

It has also been reported that in neurons acrylamide strongly influences protein synthesis and their distribution inside cells, especially the proteins engaged in building cytoskeleton (Shabana et al., 1994; Ho et al., 2002). Thus, cell differentiation occurring in granulocytes and plasmatocytes might be impeded. On the basis of different patterns of degenerations of cutaneous nerves, Ko et al. (2000) have suggested that observed discrepancies might be the effect of specific composition of cytoskeleton. The statistically significant change of plasmatocyte dimensions in acrylamide exposed housefly's larvae support an existence of similar interactions of acrylamide with cytoskeletal proteins of these cells. The morphological alterations were also observed in neuroblastoma cells exposed to acrylamide. In these cells the author has observed round or flat cell shapes and lack of protrusions (Nakagawa-Yagi et al., 2001).

The method of testing toxicity of acrylamide using the housefly's haemocytes, described in the present study, which showed similar effects of acrylamide like in mammals, can be used as a routine, cheap and easy test to evaluate toxicity of contaminations in food products for humans. Our study showed that haemocytes are sufficiently sensitive cells to be used in a health risk evaluation of different substances present in food.

5. Conflict of interest statement

The authors declare that there are no conflicts of interest.

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