ACRYLAMIDE INFLUENCE ON THE ACTIVITY OF CHOLINESTERASES, OXIDATIVE STRESS MARKERS, CASPASE-3 EXPRESSION AND MICROSTRUCTURE OF THE FOREBRAIN OF CHICKEN EMBRYOS

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ABSTRACT
The aim of this study was to examine the influence of acrylamide (ACR), a common neurotoxin, on the neuroepithelial cells utilizing in ovo model for embryonic brain development. On day five (ES), 75 eggs with living embryos were randomly divided into three groups: one control and two experimental (n=25) and injected with 0.7% NaCl and/or ACR saline solution at the dose of 30 μg or 300 μg per egg, respectively. The injections were performed directly into the amniotic fluid, and after 7 h of incubation, tissues the brain was collected. Additionally, for the brain microstructure evaluation brain tissues were obtained from 10th day embryos. Immunohistochemical localization of caspase-3 and histochemical staining demonstrated deterioration and significant damage to neuroepithelial cells and affected caspase-3 distribution/expression in brain. Results of biochemical assays showed increased activity of enzymes (SOD, AChE, BuChE) whereas ACR had no impact on the MDA concentration. This suggests that ACR has no direct impact on the caspases, and its action is an effect of SOD response to free radicals probably. ACR has an impact on the activity of cholinergic nerves and brain cytoarchitecture of the developing embryos. All of the observed effects suggests serious influence of ACR on the developing brain by several different mechanisms.

Keywords: acrylamide, neuroepithelial cells, oxidative stress, caspase-3, brain, microstructure

INTRODUCTION
Acrylamide (ACR) and its polymer, polyacrylamide, are broadly applied in laboratories and industrial processes. The polymeric form is relatively non-toxic, whereas its electrophilic monomer has been reported to pose adverse effects. The potential risk to the ACR exposure is due to the prevalence of this toxin in carbohydrate-rich processed food, tobacco smoke and occupational exposure (Smith et al., 2000). Accumulating evidence suggested that ACR induces a high risk of neuropathic alteration in humans and animals (Friedman, 2003; Riboldi et al., 2014). Acrylamide also occurs in animal feeds and can be carried over to food of animal origin. It was shown that acrylamide may enter the animal feed chain via products from the food industry such as biscuits, bread, crisps, bread, potato products, waffles and other heated by-products (Hulle et al., 2006). Electrophilic properties, allow ACR to cross the biological barriers (Von Stedingk et al., 2011; Duarte-Salles et al., 2013). Previous studies indicate that ACR influences human astrocytoma cells and nerve terminal degeneration of the central nervous system (Yu et al., 2005; Chen et al., 2013). ACR also has an impact on the cholinergic system. It was found that ACR enhanced the activity of the acetylcholinesterase (AChE) in peripheral nerves (Pennisì et al., 2013). Another study indicated declined activity of AChE in the mice brain after ACR administration (Kopańska et al., 2015). Furthermore, ACR is known to promote the induction of reactive oxygen species and depletion in anti-oxidant rates which may then lead to the neurodegenerative conditions including neuronal apoptosis of the cerebral cortex (Lopachin and Gavin 2008; Lakshmi et al., 2012; Batoryna et al., 2017). However, little is known about its influence on the development of the neural system in animal embryos. Taking the above into consideration, our study has been focusing on the ACR impact on the neuroepithelial (NE) cells, which undergo intensive proliferation in order to produce more progenitor, neuronal and macroglial cells within the central nervous system. To recognize the possible mechanism of ACR toxicity in the brain of animal embryos, we have performed measurements of antioxidative enzymes activity, cholinesterases activity, and analysis of caspase-3 expression. In ovo model has been chosen as a well-recognized and suitable model for animal embryo development studies (Stern, 2005).

MATERIAL AND METHODS
Animals and acrylamide treatment
75 fertilized hen eggs, weight 62.70 ± 6.11 g (±SD), from the commercial broiler breed parental flock of Ross 308 line, were incubated at 37.5 ± 0.5 °C (±SD), with 55% humidity within 5 days. On day five (ES) 75 eggs with the living embryos were divided into three groups (one control and two experimental). Next, the eggs were candled for the embryo localization. After that, window (about 1 cm x 1 cm) was cut in the egg shell, and the solution (50 μl volume) was injected across the choriorallantoic membrane into the amniotic fluid. Next the windows in the egg shells were covered with Parafilm® M, and the eggs were incubated for seven hours. Experimental groups (25 eggs each) were injected with 30 μg or 300 μg of ACR per egg dissolved in 0.7% NaCl. Control group of 25 eggs was injected with 0.7% NaCl. A study performed by Sörgel et al. (2002) showed that the half-life of acrylamide in the human body is 2-7 hours. The used procedure of injections of chick embryos was proposed by (Johnston et al., 1997; Dong et al., 2014). After seven hours incubation, embryos were dissected, fixed in freshly prepared 4 % (v/v) buffered (0.1 M phosphate buffer, pH = 7.6) formalin or immediately frozen and stored at -80°C until further analysis. Additionally, to observe the effect of ACR on the brain microstructure changes, part of the embryos (5 from each group) were left for the development until tenth day (10E), embryos were dissected and fixed in freshly prepared 4 % (v/v) buffered formalin. The experiment was carried out in accordance with the provisions of the National Ethical Committee for Animal Experiments.

Biochemical assay
Biochemical analyses were performed on the 12 embryos (n=12) from each group (after 7 hours administration).
Superoxide dismutase activity

The analysis of superoxide dismutase (SOD) activity was assayed according to the method described by Kono (1978) and Rice-Evans et al. (1991). The assay is based on the inhibiting influence of SOD on the reduction of cytochrome C by the superoxide anion, which is generated by xanthine/xanthine oxidase. The tissues were homogenized in ice-cold 100 mM potassium phosphate buffer (pH 7.0). The absorbance was measured at λ = 550 nm for 120 seconds using the MARCEL S330 spectrophotometer (Marcel, Poland). The results were presented as U/mg protein.

General protein concentration

General protein concentration was determined using the Bradford's method (1976). The ultraviolet spectrum for dye in acidic solution is at 405 nm and by the protein binding, the maximum moves towards longer wavelengths and occurs at 595 nm. The calibration curve was prepared with bovine albumin (BSA) in the range of protein concentration of 0.1 – 1.4 mg/mL. The measurement was performed by using the microplate reader (TECAN Sunrise) at wavelength λ = 595nm.

Malondialdehyde concentration

The concentration of the malondialdehyde (MDA) was determined according to Ohkawa (1979). The double-heating method based on the reaction of thiobarbituric acid (TBA) with MDA was used. The tissues (1mg) were homogenized in the homogenizer (CAT X360) with 10 ml of RIPA buffer (cell lysis solution reagent) containing 0.1% v/v protease inhibitor. After centrifugation (4500rpm), sodium dodecyl sulfate (SDS), trichloroacetic acid (TCA), and TBA were added to the sample and boiled. Next, the sample was cooled, centrifuged and the supernatant was placed on the microplate reader TECAN to measure the absorbance at λ = 535 nm with respect to the blank solution. The results are presented as μM/mg tissue.

Acetylcholinesterase (ACHE) and butyrylcholinesterase (BuChE) activities

For ACHE and BuChE measurements brain tissues were homogenized in ice cooled (4°C) 0.1 M sodium phosphate buffer (pH = 8) in the proportion of 1 ml buffer per 20 mg of the tissue. The supernatant obtained after centrifugation of tissue homogenate was used to measure acetylcholinesterase and butyrylcholinesterase by the Ellman's (1961) colorimetric procedures. The method base on the hydrolysis of acetylthiocholine or butyrylthiocholine iodide, where the final product is thiocholine. Thiocholine in the presence of the highly reactive dithiobis-2-nitro-benzoic acid (DTNB) reacts to generate the yellow 5-thio-2-nitrobenzoate product. The absorbance was measured at λ = 412 nm after 60 seconds. The results were presented as μM of acetylthiocholine or butyrylthiocholine iodide per g tissue/1h. Detection was performed with a MARCEL S330 spectrophotometer.

Histochemical Preparation

Sections were prepared from the whole body of the embryo. The detailed microscopic analyses were performed in the forebrain. Five microtome sections from brains of each group (7 hours ACR administration, 5, 15, 30, 45 minutes thick) were deparaffinized, rehydrated and submitted to hematoxylin or DAPI (4’, 6-diamidino-2-phenyl-indole) staining procedure. Immunohistochemical localization of caspase-3 was performed after incubation in citric buffer (pH=6.0, 75°C) and quenching endogenous peroxidase activity with 3% H2O2 solution in methanol. Nonspecific binding of the secondary antibody was blocked with 5% (v/v) normal goat serum in TBST (RT). Sections were then incubated with primary rabbit polyclonal antibody against caspase-3 (1:300) for 12 h at 4°C, followed by biotinylated goat anti-rabbit antibody (RT) and avidin-biotin-peroxidase complex and diaminobenzidine (DAB) and H2O2 solution. Light/fluorescent microscope (Nikon AZ-100 with light NIS ELEMENTS AR) and ImageJ software (version 2.0.0-rc-43f1.51p) were utilized to calculate the ratio of caspase-3-positive cells. Each slide was examined in five random areas. To estimate ACR effects on the further brain development brain tissues of embryos exposed to ACR up to E10 were dissected to perform hematoxylin and eosin staining according to Fisher et al. (2008).

Experimental Materials

The primary rabbit polyclonal antibody against caspase-3 (1:300), biotinylated goat anti-rabbit antibody (RT), avidin-biotin-horseradish peroxidase complex and diaminobenzidine were obtained from Abcam (Cambridge, UK). Reagents for superoxide dismutase (SOD), malondialdehyde (MDA), acetylcholinesterase (ACHE) and butyrylcholinesterase (BuChE) measurements, cytochrome C, xanthine oxidase, thiobarbituric acid, protease inhibitor, rabbit immunoperoxidase reaction buffer (RIPA), sodium dodecyl sulfate (SDS) for acetylthiocholine iodide, butyrylthiocholine iodide, DTNB (Ellman reagent) were supplied by Sigma Aldrich (Saint Louis, Missouri, USA), hematotoxin (Stamen, Poland) and eosin (Stamen, Poland).

Statistical analysis

The dependent variables were checked for their distribution and homogeneity using Shapiro-Wilk and Levene tests respectively. The results were compared by one-way ANOVA followed by Tukey’s test. The significance level was established at p<0.05.

RESULTS AND DISCUSSION

The main protein of cholinergic synapses in the brain and neuromuscular connections is acetylcholinesterase. The function of the enzyme is to regulate the concentration of acetylcholine in synapses. Our study, confirm the hypothesis of ACR neurotoxicity. Increased activity of cholinesterases may have a significant influence on the brain of animal embryos. Coehard and Coltey (1983) demonstrated that cholinesterase activity, was present at all axial levels in presynaptic crested cells. This suggests that its role is the regulation of synaptic transmission. Furthermore, AChE plays a crucial role during early vertebrate development, in the example; neuromuscular development (Behra et al., 2002), cell differentiation and network formation, early embryonic (limb) development (Lay et al., 2013).

Other proposed mechanism of ACR neurotoxicity is associated with oxidative damage (Lopachin and Lehning, 1994). One of the defense mechanisms against free radicals is a triad of antioxidant enzymes, including superoxide dismutase, glutathione peroxidase, and catalase (CAT). SOD catalyzes the dismutation of the superoxide anion to the hydrogen peroxide which is decomposed to water and oxygen by other enzymes (Meister, 1998). Redox imbalance is detrimental to the brain function and leads to the oxidative stress and DNA damage. Therefore, neurons are the first cells affected by free radicals in the brain (Kaluzna-Czaplinska et al., 2008). To estimate the oxidative damage, we measured MDA concentrations, a secondary product which is used as convenient marker for lipid peroxidation (Ayala et al., 2014). No differences in MDA concentration (Fig. 2), observed in our studies, suggest that the damage of neuroepithelial cells was not related with lipids peroxidation. Moreover, increased SOD activity (One way ANOVA F (2, 18)=5.4961, p<.05, Fig.1) accompanied by higher AChE and BuChE activity could also have an impact on MDA level, because of the effective protection against oxidative stress by these enzymes (Liu et al., 2001). However, the distinctiveness between our results and the results of other research might have been caused by the diverse periods of ontogenesis, low doses of ACR and short time of exposure to ACR in our research. High activity of SOD and no symptoms of lipids peroxidation, may suggest a genotoxic mechanism of ACR influence on the nervous system. The main factors that can cause neuronal death are the high level of free radicals, and the increased activity of cholinesterases, as well as the decreased level of SOD activity. Decreased activity of SOD may increase the AChE and BuChE activity, which is associated with the increased activity of caspase-3 and ACR on cholinesterases, observed in our results, comparing to other authors, may suggest a genotoxic mechanism of ACR neurotoxicity. Increased activity of cholinesterases may have a significant influence on the brain of animal embryos. Coehard and Coltey (1983) demonstrated that cholinesterase activity, was present at all axial levels in presynaptic crested cells. This suggests that its role is the regulation of synaptic transmission. Furthermore, AChE plays a crucial role during early vertebrate development, in the example; neuromuscular development (Behra et al., 2002), cell differentiation and network formation, early embryonic (limb) development (Lay et al., 2013).
protease-activating factor 1 (Apaf-1) and cytochrome c (CytC) is responsible for the autocatalytic activation of an initiator caspase-9 which activates executioner caspase-3 (Chai and Shi, 2013). Caspase-3 appears to be the major executioner caspase involved in the brain neuronal apoptosis. Suppression or stimulation of apoptotic capability is essential to normal brain development (Porter and Jänicek, 1999).

In our results, the antibodies specific for caspases-3 were detected in all samples. The calculation using ImageJ software and statistical analyses has shown that there were statistically significant differences in expression of caspase-3 between control and experimental groups (One way ANOVA, F (2, 26)=19.896, p<0.0001). After ACR administration the number of caspase-3 positive cells substantially decreased (Fig. 4). In control embryos (Fig. 5A), caspase-3 was evenly distributed throughout all the layers of neuroepithelial cells, whereas in embryos exposed to ACR (Fig. 5B, 5C) the enzyme positive cells concentrated in the mantle layer of the forebrain vesicle. Similarly, to our results, in vitro studies carried out on rat primary astrocytes and human astrocytoma cell lines showed decreased expression of procaspase-3, -8, and -9 after exposure to ACR (Lee et al., 2014). Although, in most previous studies, acceleration of the apoptotic processes, driven by ACR intoxication was observed (Sumizawa and Iguš 2007; 2009). Zhong et al., (2016) indicated that caspase-dependent neuroepithelial cell apoptosis enhanced by oxidative stress was reduced by superoxide dismutase overexpression. In addition, Sugawara et al., (2002) indicated that the overexpression of SOD reduced oxidative stress, thereby attenuated the mitochondrial release of cytochrome C and second mitochondria-derived activator of caspases, which resulted in a lower expression of caspases and consequently diminished rate of apoptosis. In our studies inhibition of caspases at E5 resulted in increased number of neural cells in brain hemispheres at E10, which points to the disturbances in the selection of the neuron cells in embryos exposed to ACR (Fig. 6A, 6B). Similar relation between superoxide dismutase activity and caspases was indicated in the brain of ischemic adult mice and in human umbilical vein endothelial cells (Dimmel et al., 1999; Noshita et al., 2001). Thus, it seems that in our research the attenuated caspase-3 activity could be associated with the enhanced activity of SOD. It is also reasonable to suggest that increased SOD activity resulted from the higher rate of superoxide anion formation in brains of embryos exposed to ACR (Fukai and Ushio-Fukai, 2011). Moreover, Kim et al., (2008) observed that AChE inhibited activation of caspase-3 in hypoxia and the same effect we observed in our results. We have also observed large extracellular intervals/spaces as well as a loss of intermitotic neuroepithelial cells and neuroblasts in the external zone of the mantle layer of the forebrain vesicle (Fig. 5G). Numerous cells with fragmented and pyknotic nuclear material were evident. At the dose of 300 μg/egg (Fig. 5H), larger extracellular intervals were noted, (Fig. 5E, 5F) in addition to a massive loss of neuroblasts and intermitotic neuroepithelial cells. Moreover, swollen cells in the wall of the forebrain vesicle were observed. Our study manifested prevalent pyknotic nuclei in both experimental groups (Fig. 5G, 5H) in comparison to control group (Fig. 5F). Additionally, in the present study occurrence of macrophages in the proximity of the external limiting membrane was observed (Fig. 5G). These results confirm the observations of the neuronal structure damage after ACR intoxication, indicated by Zhang et al., (2017).

**Figure 1** The influence of *in ovo* ACR application on the SOD activity (U/mg protein) in the 5-day old chicken embryo forming brain. Difference significant in comparison to control at p<0.05 (**). Bars represent mean value of SOD activity. Vertical lines indicate standart deviation ranges (SD).

**Figure 2** The influence of *in ovo* ACR application on the MDA concentration in the 5-day old chicken embryo forming brain (µM/mg tissue). There was no statistical differences between groups. Bars represent mean value of MDA concentration. Vertical lines indicate standart deviation ranges (SD).

**Figure 3** The influence of *in ovo* ACR application on the cholinesterases AChE and BuChE activities (µM iodide ATCh, BuChE/g/h). Difference significant in comparison to control at p<0.05 (**), p<0.01(***), p<0.001(****), p<0.0001(****). Bars represent mean value of AChE and BuChE activity. Vertical lines indicate standart deviation ranges (SD).

**Figure 4** The influence of *in ovo* ACR application on the number of caspase-3 positive grains in the 5-day old chicken embryo forming brain (relative units). Difference significant in comparison to control at p<0.05 (**), p<0.01(***), p<0.001(****), p<0.0001(****). Bars represent mean value of caspase-3 positive cells. Vertical lines indicate standart deviation ranges (SD).
Figure 5 The effect of ACR exposure on the forming chick embryo brain. Section through wall of forebrain vesicle of the 5-day old chicken embryo.


Figure 6 The cross section of the brain, left upper region brain hemisphere of the 10-day old chicken embryo.

A: Control group (E10). Staining with hematoxylin & eosin. Free space located on the right side of the photo is the ventricle of the left hemisphere. B: ACR dose 300 μg/egg (E10). Staining with hematoxylin & eosin. Increased density of neural cells visible in brain of embryos exposed to ACR in comparison with control embryos (B).

CONCLUSION

Our experiment demonstrated significant damage to neuroepithelial cells in the brain of developing embryos exposed to ACR. Taking into consideration that no differences in MDA concentrations were observed, the mechanism of the neuroepithelial cells damage seems not related with lipid peroxidation process. The observed effects, i.e., raised deterioration of neuroepithelial cells and modified caspase-3 distribution/expression in the forming brain, may significantly influence the central nervous system development of embryos as well as their post hatch performance. It seems that ACR has no direct impact on the caspases, and its action is an effect of SOS response to free radicals generated by ACR and increased activity of cholinesterases.

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