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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 (E5), 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 by-products from the food industry such as biscuits, bread, crispbread, potato products, waffles and other heated by-products (**Halle *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 (**Pennisi *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 (E5) 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 1cm x 1cm) was cut in the egg shell, and the solution (50 µl volume) was injected across the chorioallantoic 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 absorption spectrum for dye in acidic solution is at 465 nm and after 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 butyryltiocholine 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 butyryltiocholine iodide per g tissue/h. 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 perfomed in the forebrain. Five microtome sections from five embryos form each group (7 hours ACR administration), n=5, (5 μm thick) were deparaffinized, rehydrated and submitted to hematoxyline 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% H_2O_2 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-horseradish peroxidase complex. Colour reaction was developed using diaminobenzidine (DAB) and H_2O_2 solution. Light/fluorescent microscope (Nikon AZ-100 with light NIS ELEMENTS AR) and ImageJ software (version 2.0.0-rc-43/1.51p) 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, radioimmunoprecipitation assay buffer (RIPA), sodium dodecyl sulfate (SDS), acetylthiocholine iodide, butyryltiocholine iodide, DTNB (Ellman reagent) were supplied by Sigma Aldrich (Saint Louis, Missouri, USA), hematoxylin (Stamar, Poland) and eosin (Stamar, 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 conduction of nerve impulses by fast hydrolysis of the neurotransmitter – acetylcholine **(Jbilo *et al*. 1994)**. The expression of the cholinergic system during embryonic development is a common occurrence. In mammals, AChE maintains high concentration during maturation and development of the placenta and the fetus. BuChE transcripts appeared before AChE transcripts early in development. In chick embryos, BChE is transiently expressed during the late stages of cell proliferation, whereas AChE expression is associated with differentiating cells withdrawn from the mitotic cycle **(Layer, 1990; Layer And Sporns, 1987). Kopańska *et al.* (2017)** indicated that ACR administration resulted in a significant decrease in AChE activity in different brain structures of adult Swiss mice. In rats, ACR also diminished AChE and ATPase activity in the cerebellum of mothers and their suckling pups (**Ghorbel *et al*., 2016**). **Shrivastava *et al.* (2018)**, observed significant inhibition of AChE activity in the brain after ACR administration in adult Wistar female albino rats. In contrasts, we have observed a significant increase in the activity of BuChE and AChE. Acetylcholinesterase (one way ANOVA F (2, 12)=23.250, p<.0001) and butyrylcholinesterase (one way ANOVA F(2, 12)=21.748, p<.001) activity was a significant increase in groups treated with ACR (Fig. 3) in comparison to the control. Different effect of ACR on cholinesterases, observed in our results, comparing to other authors, may be related to the specificity of physiological conditions in developing embryos. Moreover, in most of the research presented above, ACR total exposure were significantly higher than in our experiment. Nevertheless, our results confirm the general concept that disturbances of synapse functioning underlies the mechanism of ACR neurotoxicity. Increased activity of cholinesterases may have a significant influence on the brain of animal embryos. **Cochard and Coltey (1983)** demonstrated that cholinesterase activity, was present at all axial levels in presumptive crest cells of the neural folds, soon after closure of the neural tube. 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 **(Layer *at 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 neurons due to the large concentrations of unsaturated fatty acids in neuronal membranes. Therefore, neurons are the first cells affected by free radicals in the brain **(Kałużna-Czaplińska *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 neuroepithelium. This could be associated with the immense genotoxic properties of the ACR metabolite – glycidamide and its ability to create adducts with hemoglobin and DNA **(Lopachin and Gavin, 2012; De Lima *et al*., 2016)** - although this is only a hypothesis and needs further research.

During the development and maturation of the nervous system, a significant amount of neuron cells of the original population undergo apoptosis. Genetically programmed death, through the selection of the neuron cells, leads to the death of almost half of them. **(Yakovlev *et al*., 2001)**. In chickens, neuronal cell death affects differentiated ganglion cells with a peak at approximately embryonic day tenth (E10). In the earlier distinct phase of development, apoptosis occurs with a peak at approximately E5 and affects cells other than fully differentiated neurons **(De La Rosa and De Pablo, 2000)**. For that reason, in our study, embryos were treated on day five of development. Apoptosis is initiated by the activation of a cascade of caspases. Apoptosome, the multiprotein complex between apoptotic

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 Jänicke, 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<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 Igisu 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 **(Dimmeler *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)**.

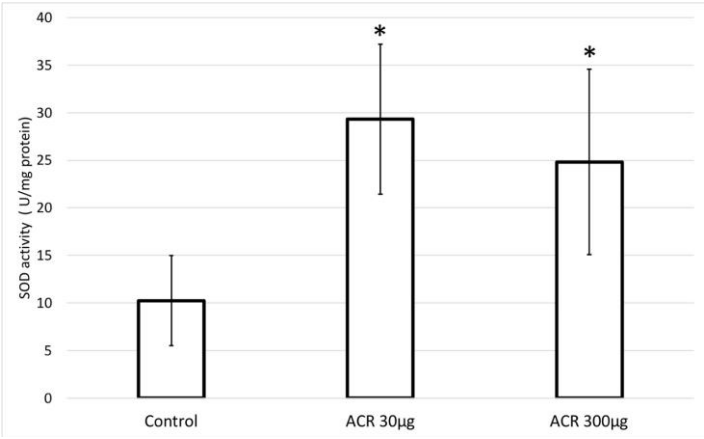

Figure 1 The influence of *in ovo* ACR application on the SOD activity (U/mg protein) in the 5-day old chicken embryo forming brain. Difference significant in comparison to control at p<0.05 (*),. Bars represent mean value of SOD activity. Vertical lines indicate standart deviation ranges (SD).

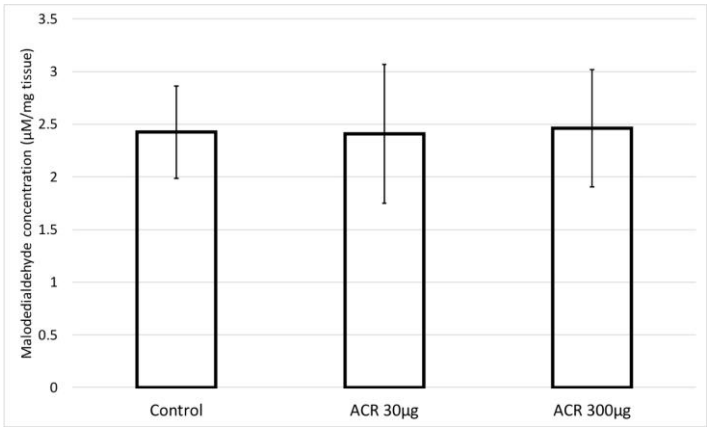

Figure 2 The influence of *in ovo* ACR application on the MDA concentration in the 5-day old chicken embryo forming brain (µM/mg tissue). There was no statistical differences beetwen groups. Bars represent mean value of MDA concentration. Vertical lines indicate standart deviation ranges (SD).

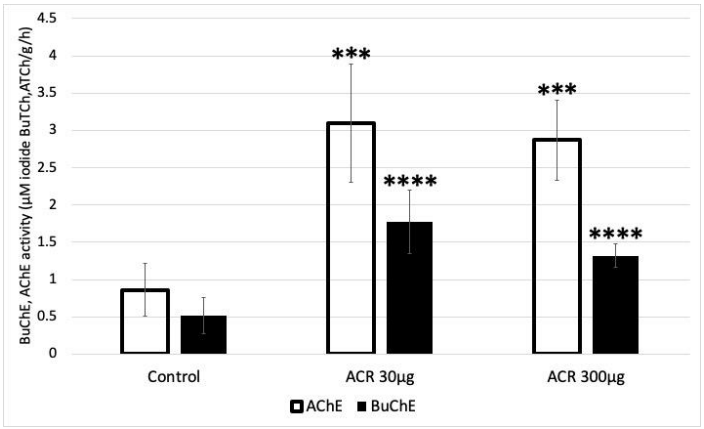

Figure 3 The influence of *in ovo* ACR application on the cholinesterases AChE and BuChE activities (µM iodide ATCh, BuChE/g/h). Difference significant in comparison to control at p<0.05 (*), p<0.01(**), p<0.001(***), p<0.0001(****). Bars represent mean value of AChE and BuChE activity. Vertical lines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

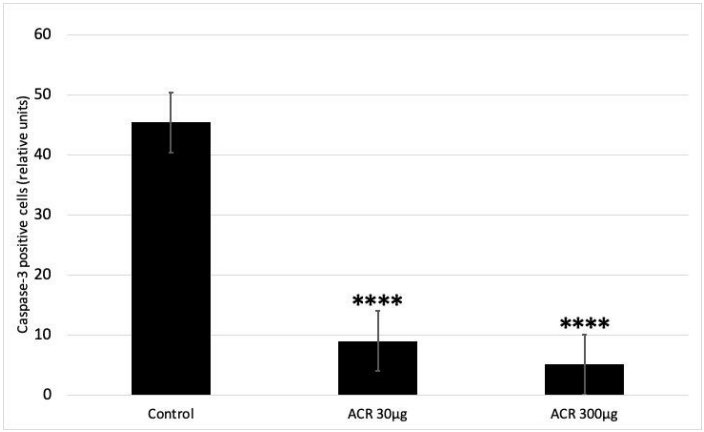

Figure 4 The influence of *in ovo* ACR application on the number of caspase-3 positive grains in the 5-day old chicken embryo forming brain (relative units). Difference significant in comparison to control at p<0.05 (*), p<0.01(**), p<0.001(***), p<0.0001(****). Bars represent mean value of caspase-3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

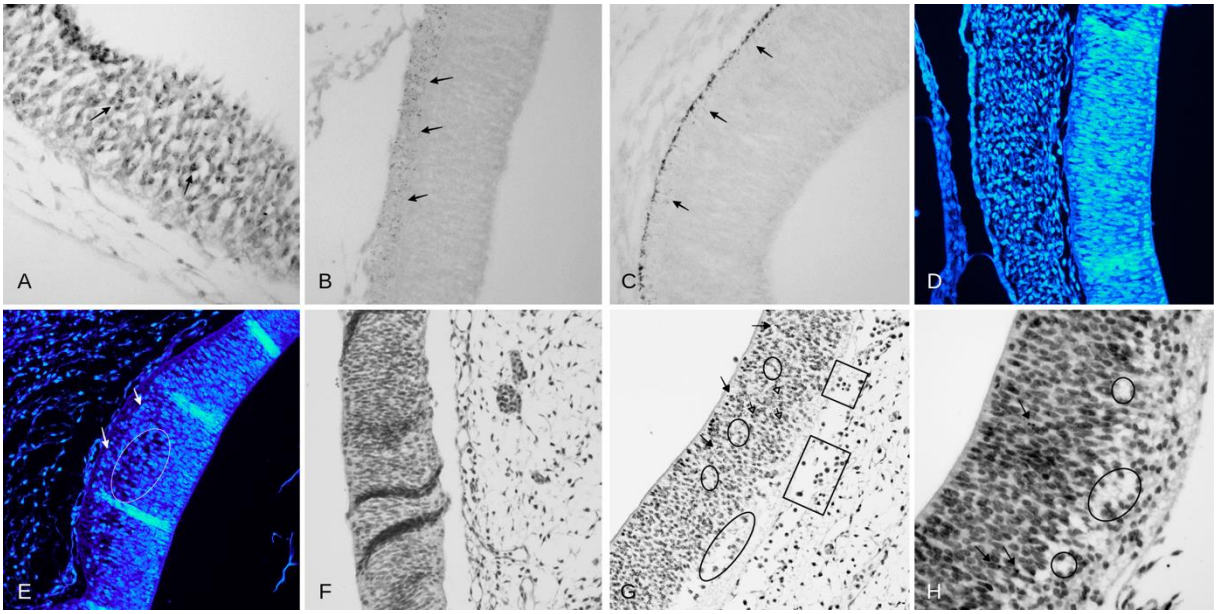

Figure 5 The effect of ACR exposure on the forming chick embryo brain**.** Section through wall of forebrain vesicle of the 5-day old chicken embryo .
A: Control group. Immunohistochemical staining for caspases 3 (primary rabbit polyclonal antibody against caspase-3). The positively stained material is evenly distributed throughout the whole layers of cells – arrows (×400). B: ACR dose of 30 µg/egg mass. Immunohistochemical staining for caspases-3. The positively stained material is distributed at the outer 3-4 layers of vesicle wall-arrows (×200). C: ACR dose of 300 µg/egg. Immunohistochemical staining for caspases 3. The positively stained material is concentrated in large amounts in the mantle layer of cells-arrows (×200). D: Control group. DAPI staining for nuclear material (×200). E: ACR dose of 30 µg/egg. DAPI staining. Massive loss of intermitotic neuroepithelial cells and neuroblasts visible in the mantle layers – ellipses. Swollen cells visible in the outermost layer – arrows (×200). F: Control group. Staining with hematoxylin & eosin (×200). G: ACR dose of 30 µg/egg. Staining with hematoxylin & eosin. Large extracellular spaces and loss of cells from mantle layer – ellipses. Pyknotic cells – full arrows. Numerous cells with vacuoles – empty arrows. Numerous macrophages, mostly located next to the external limiting membrane– rectangles (×200). H: ACR dose of 300 µg/egg (E5). Staining with hematoxylin & eosin. Massive loss of epithelial cells in central and mantle layers – ellipses. Pyknotic cells – arrows (×400)

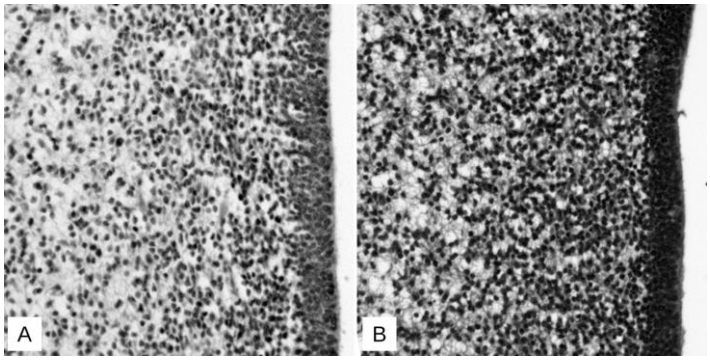

Figure 6 The cross section of the brain, left upper region brain hemisphere of the 10-day old chicken embryo.

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 SOD response to free radicals generated by ACR and increased activity of cholinesterases.

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