Developmental neurotoxicity of bendiocarb

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Introduction

Pesticides are biologically active chemicals, which have been thoroughly tested for safety and usefulness, released for agricultural use (Holovska et al., 2011). The annual application of the synthetic pesticides to food crops in the European Union exceeds 140.000 tonnes, an amount that corresponds to 280 grams per EU citizen per year. Thus, many pesticides such as organophosphates, carbamates and pyrethroids are widely used in agriculture and households (Bjørling-Poulsen et al., 2008). Carbamate insecticides have different degrees of acute oral toxicity (Costa et al., 2008). No epidemiological studies of developmental neurotoxicity of carbamates in humans could be found, and data from animal experiments are very sparse as well (Bjørling-Poulsen et al., 2008). Agrochemicals, including pesticides, are being used in increasing amounts in agriculture and are therefore potential environmental contaminants which may affect a variety of biological systems. The pesticide residues directly affect the embryos, disturbing their normal development and causing patho-physiological and morphological changes (Petrovova et al., 2010).

Animal models play a crucial role in fundamental and medical research. Progress in the fields of drug study, regenerative medicine and cancer research among others are heavily dependent on *in vivo* models to validate *in vitro* observations, and develop new therapeutic approaches. However, conventional rodent and large animal experiments face ethical, practical and technical issues that limit their usage in research area. The chick embryo represents an accessible and economical *in vivo* model, which has long been used in developmental biology, gene expression analysis and loss/gain of functional experiments (Rashidi and Sottile, 2009).

Chick embryo is a popular model for developmental pharmacological and toxicological studies. The concordance of data from CHEST (Chick Embryotoxicity Screening Test) and mammals is excellent, and it avoids potentially confounding effect of different maternal metabolism between species by allowing for separate testing of human-relevant metabolites (Jelinek, 1977). Its development takes place outside the mother body, and therefore the effect of the substance is observed directly on the embryo itself, without affecting mother's metabolism (Petrovova et al., 2009) as well as immune response. The immune system is developed in later stage of development of the chick embryo (8–12 day of incubation; Baiguera et al., 2012). Given the absence of maternal metabolism, it requires considerably smaller amounts of administered substances per embryo, which is particularly useful for testing rare or expensive compounds, or when maternal toxicity is of concern.

The nervous system of the chick embryo is formed from neural plate and the neural crest. At 2 ED the neural tube possesses two layers, the *ependyma*, which contains a large number of mitotic cells and the *marginal layer*. By 3 ED the *mantle layer* is also recognizable. Neuroblasts are visible from about 2 ED in the ventro-lateral part of tube. By 3 ED spinal nerves have developed and by 3–4 ED regions of grey and white matter are recognizable. Dorsal and ventral horns can be seen in the grey matter from 7 ED, and glial cells in the white matter. During the following days the spinal cord becomes larger in transverse section and there is a change in shape of the lumen from a longitudinal slit to an almost square or round shape (Bellairs and Osmond, 2005).

Acetylcholine (ACh) is a major excitatory neurotransmitter in the nervous system of vertebrates and invertebrates, and is synthesized from acetyl coenzyme A and choline by the enzyme choline acetyltransferase. Presynaptic choline transport supports ACh production and release, and cholinergic terminals express a unique transporter critical for neurotransmitter release. Neurons cannot synthesize choline, which is ultimately derived from the diet and is delivered through the blood stream (Amenta & Tayebati, 2008). ACh plays regulatory roles throughout ontogenesis, including stages prior to development of the nervous system (Buznikov et al., 1996). Accumulated evidence suggests that ACh plays a key role in regulation of morphogenetic cell movements, cell proliferation, growth, and differentiation in species as diverse as echinoderms, insects, worms, avians, rodents, and humans (Lauder & Schambra, 1999). Developing animals are more sensitive than adults to acute cholinergic toxicity from anticholinesterases, including organophosphate and carbamate pesticides, when administered in a laboratory setting. It is also possible that these agents adversely affect the process of neural development itself, leading to permanent deficits in the architecture of the central and peripheral nervous systems (Slotkin, 2004). Prenatal exposure to organophosphate and carbamate pesticides could have adverse effects on neural development by interfering with the mor-

phogenic function of acetylcholinesterase (AChE). Accumulating evidence indicates that AChE has extrasynaptic functions during neural development (Bigbee et al., 1999). This idea was initially based on *in vivo* observations that AChE is transiently expressed by neurons throughout periods of axonal outgrowth prior to synaptogenesis, a period during which the classical cholinolytic role for AChE in terminating nervous transmission is unnecessary. In the chick, transient AChE expression occurs in developing spinal cord neurons, which coincides with axonal outgrowth from these cells (Weikert et al., 1990). In the peripheral nervous system, AChE is transiently expressed by developing dorsal root ganglion neurons and later in their axons and growth cones in the spinal cord. The first trimester of fetal development is the most sensitive target for adverse effects of drugs or chemicals may make developing neurotransmitter system especially vulnerable to environmental neurotoxins, such as pesticides, designed to target receptors for these neurochemicals in lower organisms (Slotkin, 1999).

As well as regions of high proliferation there are also areas that have a high rate of cell death. Probably in all embryonic tissues there is a continuous loss of cells through death, but in certain parts of the developing body the death rate outstrips the proliferative rate. This means, that just a same region becomes enlarged by rapid cell division, others become eroded away. Apoptosis is also known as "programmed cell death" because in many cases the patches of cells die in a particular location of the embryo at a specific time in development and play an important role in morphogenesis. Region of cell death plays a specific role in the shaping and patterning of organs. Apoptotic cells are, however, found in normal embryos even as early as gastrulation (Bellairs, 1961) and after that in many well-defined sites in the differentiating tissues, e.g. in the mesonephros, in the heart, in the sclerotome, in the tail bud, branchial arches, lateral body wall (Hirata and Hall, 2000) and also in the nervous system and neural crest. In most of these examples cell death is focused on a highly localized region and occurs within a restricted period of time (Bellairs and Osmond, 2005). Hirata and Hall (2000), who have reviewed the temporo-spatial patterns of cell death from developmental stages 1–25 (according to Hamburger and Hamilton, 1951) have concluded that cell death is a feature of development at all these stages but that there are changing patterns, depending on the specific stages of growth, differentiation and morphogenesis.

In order to assess to the fullest extent the possible embryotoxic potential, we performed the study of bendiocarb effects in the chick embryo. We observed the toxicity (mortality and weight of survived embryos – LD_{50}) of the cholinesterase inhibitor bendiocarb and the associated occurrence of malformations during various developmental stages (embryonic days 2–5 and 10) and with different bendiocarb concentrations (8–1600 μg/egg). Subsequently, we observed the organ toxicity as well as the programmed cell death (apoptosis) and total number of dead cells in the central nervous system (CNS) after bendiocarb treatment.

Materials and methods

Eggs

The number of 651 fertilized White Leghorn chicken eggs were purchased from the animal facility of the Institute of Molecular Genetics (Koleč, Czech Republic) and delivered via courier in a temperature controlled manner to ensure egg viability and quality. They were incubated without storage blunt end up in a forced-draft constant-humidity incubator at 37.5°C with continuous rocking and relative humidity 60% until embryonic days (ED) 2–10 of the (21–day) incubation period. According to Hamburger and Hamilton (1951), embryonic days from the ED 2 to ED 10 represent the 19^{th} to 36^{th} stages of development. Embryos were observed during incubation and dead, growth retarded or dysmorphic individuals at the time of treatment were excluded from further study.

Application of bendiocarb

The bendiocarb (2,2-dimethyl–1,3-benzzodiol–4-yl-*N*-methyl carbamate, Bendiocarb Tech, 98.9%, Bayer, Germany) was dissolved in acetone and diluted with sterile water for injection to obtain the required concentrations.

At embryonic days 2, 3, 4, 5 and 10, the eggs were opened by the modified „window technique" (Jelinek, 1977). The blunt end of eggs was cleaned with 70% alcohol and covered by a transparent adhesive tape (Sedmera et al., 2002). Subsequently, using serrated scissors (FST 14071–12), an opening was cut for application of the respective doses of bendiocarb. Bendiocarb was dissolved in acetone and diluted with sterile water for injection to obtain the required concentrations (8–1600 μg/200 μl/egg). The tested solution was applied directly over the embryo on the top of inner shell membrane (*membrana papyracea*). Based on our past experience, the doses used for administration through the papyraceous membrane directly over the embryo and its developing vasculature were the same as for intraamniotic route (Sedmera and Thompson, unpublished observations), but there is less procedure-related mortality and it is technically more simple (easier windowing and closing, yet keeping the advantage of visual control and possibility to exclude grossly abnormal embryos). The application dose per one egg was 200 μl, with acetone concentration equal to 10 μl/200 μl of application dose. Controls received the same volume of solvent alone – 10 μl of acetone in 200 μl of water for injection. The ranges of concentration as well as the total number of embryos and the days of application are listed in Table 1. After application of solutions the eggshell openings were covered with an electrical insulation adhesive tape and the chicken embryos were transferred to a thermostat and incubated under standard incubation conditions (temperature 37.5 ± 0.5°C, 60% relative humidity, without turning or adding CO_2; Sedmera

and Novotná, 1994). The embryos were observed during incubation and those which died were eliminated from the experiment.

Cytotoxicity of the CNS was observed by application of bendiocarb on ED 3 (early developmental stage) and ED 10 (later developmental stage).

Processing of the chick embryo

At the time of bendiocarb treatment on ED 2–5 the chick embryos were removed on ED 9. At the time of bendiocarb application on ED 10 the chick embryos were removed from eggs on ED 17. The survival chick embryos were removed from the eggs using a crook, weighed and examined under the stereomicroscope (Olympus SZX2) for monitoring of the malformation spectrum (malformation of the eye, beak, palate, body wall and limbs).

Light microscopy – organ toxicity

After the examination under the stereomicroscope (Olympus SZX2) the embryos were fixed for 24 hours in Dent's solution (20% dimethyl sulphoxide and 80% methanol) and processed by a standard way for histological examination. Part of the neck was separated from the fixed chicken embryos (exposure at ED 3 and ED 10). The respective parts of embryos were embedded in paraffin and after 24 hours a microtome (Leica RM 2265) was used to cut sections of thickness 10 μm.

To observe the microscopic changes in the CNS, part of the sections was stained with haematoxylin-eosin. The microscopic examination was carried out under optical microscope Olympus BX 51 using a dry objective with 60x magnification. Pictures were taken subsequently using a digital camera DP 70 and Cell P (Olympus) software.

Fluorescence microscopy – caspase activity

The remaining part of the sections was stained immunohistochemically for observation of caspases activity. The caspases activity was observed in the CNS by means of primary murine monoclonal antibody IgG 1–Caspase-3/CPP32 (BD Pharmingen) and secondary antibody conjugated with Rhodamine Red dye (Jackson ImmunoResearch). To visualize the nuclei in the CNS, respective sections were stained with Hoechst 33258 dye (Calbiochem). The Rhodamine Red-conjugated antibody was red under a fluorescent microscope when using a suppression filter (465 nm) while Hoechst 33258 stain was blue when using an excitation filter (420 nm). Autofluorescence in the fluorescein channel was used for tissue contrast. Microscopic examination was carried out by means of a fluorescence microscope Leica using a dry objective with 60x magnification.

Confocal microscopy – detection of total dead cells

In a separate group of 11 embryos treated with 400 μg of bendiocarb on ED 3 and 5 controls, the sampling was performed at 24 and 48 h intervals for the purpose of whole-mount detection of dead cells using Lysotracker Red (Invitrogen, USA; Schaefer et al., 2004). After staining, the embryos were fixed with 4% paraformaldehyde (Sigma-Aldrich, Germany) in phosphate-buffered solution (PBS; NBS Biologicals, England) for 24 h at 4°C, rinsed in PBS, dehydrated through graded ethanol (Sigma-Aldrich, Germany) series and cleared in benzyl alcohol (99%) – benzyl benzoate (99%, Sigma-Aldrich, Germany; in mixing rate (1:1) for examination on a confocal microscope) (Miller et al., 2005). Validity of using Lysotracker Red for whole-mount detection of cell death was verified using bromodeoxyuridine (BrdU; 99%, Fluka, Switzerland) in the positive control group. An applied dose of 5 μg bromodeoxyuridine is considered to be embryotoxic on ED 3–5, causing alterations of programmed cell death and deviation of limb development (Sedmera and Novotna, 1994). Images acquired in the green and red channels on a Leica SPE confocal microscope were processed using Adobe Photoshop.

Statistical analysis

Statistical comparison of different groups was performed using the statistical software GraphPad Prism 5. Subsequently, a paired t-test was used to compare the results between treated and control groups. Value of $p<0.05$ were considered significant.

Tab. 1. Application doses, embryonic days (ED) of application and number of embryos (N)

	ED	dose (μg)	N
Embryotoxicity	2	control	35
		8	20
		80	22
		200	13
		400	11
		800	21
		1200	22
	3	control	36
		16	24
		160	19
		500	15
		1000	22
		1600	50

Embryotoxicity	4	control	23
		16	17
		160	18
		500	19
		1000	20
		1300	21
		1600	12
	5	control	40
		80	22
		160	21
		320	21
		500	23
		1000	19
		1600	21
	10	control	12
		800	17
		1600	15
Organ toxicity	3	control	36
		500	15
	10	control	12
		800	17

Results

Embryotoxicity

Total embryotoxicity of a single dose of bendiocarb after application on ED 2, 3, 4, 5 and 10 was investigated on the sampling days (ED 9 and 17). The embryolethality (expressed as LD_{50}; Tab. 2) decreased with increasing age (Fig. 1), except for ED 3, when the LD_{50} was the lowest. The embryolethality after bendiocarb application on ED 10 could not be determined because of solubility limits (200 g/l of acetone, but lower in mixture with water for injection; too much of concentrated acetone is toxic to the embryo). In general, administration of bendiocarb resulted in a small decrease in embryonic weight, with a clear correlation of dose at later developmental stages (ED 5 and 10).

Similar to findings in adult mammals and birds, the embryotoxicity of a single dose is rather low in the chick, with the youngest stages being the most sensitive (Tab. 2; calculated LD_{50} doses based on the embryonic weight are in the range 20–200 g kg^{-1}; considering the whole ~30 g egg as a distribution space, the range would be 24–924 mg kg^{-1} according to stage). It is unlikely that such doses or concentration would be achieved during environmental exposure; however, it does not necessarily mean that even lower concentrations could not cause harm to more sensitive individuals.

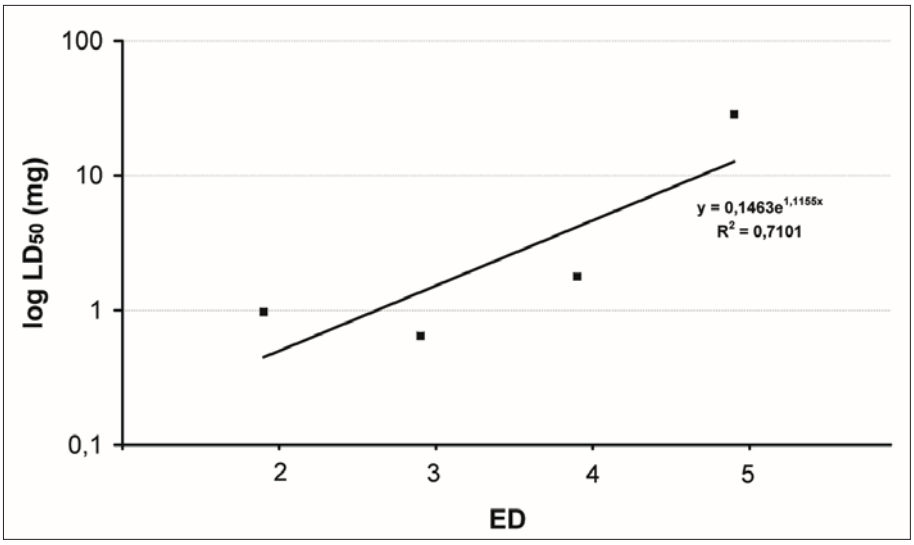

Fig. 1. LD_{50} of bendiocarb increased with stage of development

The malformations were observed sporadically in both treated and control groups, with overall frequency below 2% against mortality (30%). Examples of malformations included defects of body wall, microphthalmia, anophthalmia, cleft beak, haemorrhagic spots and general growth retardation (Fig. 2). No specific pattern of malformations was observed among the treated embryos, irrespective of the dose and embryonic stage at its application. There were no gross anomalies or overt growth retardation among the survivors. We thus conclude that bendiocarb does not possess a significant teratogenic potential, at least in the avian embryo. Nevertheless, large doses that would impair maternal metabolism could cause secondary problems to the developing embryo or fetus in mammals.

To discern potentially subtle toxic effects that are compensated later on by increased cell proliferation, we performed whole-mount staining with Lysotracker Red to detect dead cells in the whole embryo 24 h (ED 4) and 48 h (ED 5) after application on ED 3 (Fig. 3). There were no gross anomalies or overt growth retardation among the survivors. The areas of programmed cell death, revealed by Lysotracker Red staining, were observed in the developing eye, face (branchial region), limbs and tail. There was a mild increase in the intensity of staining between bendiocarb-treated and control embryos at 24 h but no difference at 48 h sampling interval. The extent of cell death was remarkably increased in freshly dead treated embryos at 24 h. The validity of using Lysotracker Red for whole-mount detection of cell death was verified using bromodeoxyuridine (5 μg of BrdU) in a parallel experiment.

Tab. 2. Bendiocarb embryotoxicity at different stage of development; * Wet weight of embryos sampled on ED10, except for application at ED10 when sampling was done at ED17; $p<0.05$ values considered statistically significant are in bold

ED	Dose (µg)	N	Dead	Mortality (%)	Malformed	Mean weight (g)*	Weight SD	LD_{50} (mg/egg)
2	0	35	5	14	3	1.355	0.272	
	8	20	0	0	0	**1.546**	0.218	
	80	22	1	5	1	1.399	0.378	
	200	13	5	38	0	1.363	0.119	**0.973**
	400	11	5	46	0	1.305	0.081	
	800	21	7	33	0	1.196	0.245	
	1600	22	16	73	0	1.304	0.267	
3	0	36	4	11	0	1.446	0.165	
	16	24	6	25	1	1.421	0.183	
	160	19	2	11	0	1.536	0.111	**0.646**
	500	15	10	67	0	1.312	0.131	
	1000	22	18	82	0	1.331	0.139	
	1600	50	47	94	0	1.544	0.031	
4	0	23	2	9	1	1.139	0.201	
	16	17	1	6	0	1.162	0.151	
	160	18	1	6	0	1.196	0.119	**1.783**
	500	19	0	0	0	1.225	0.134	
	1000	20	10	50	0	1.115	0.113	
	1300	21	6	29	1	1.188	0.163	
	1600	12	6	50	0	1.145	0.129	
5	0	40	4	10	0	1.496	0.126	
	80	22	9	41	0	1.445	0.187	
	160	21	11	52	1	1.635	0.220	**28.571**
	320	21	10	48	1	1.364	0.152	
	500	23	1	4	0	1.306	0.133	
	1000	19	1	5	0	1.394	0.182	
	1600	21	1	5	0	1.382	0.151	
10	0	12	0	0	0	17.25	1.324	
	800	17	3	18	3	14.86	2.543	**not determined**
	1600	15	5	33	0	15.30	1.337	
Total		**651**	**197**		**12**			

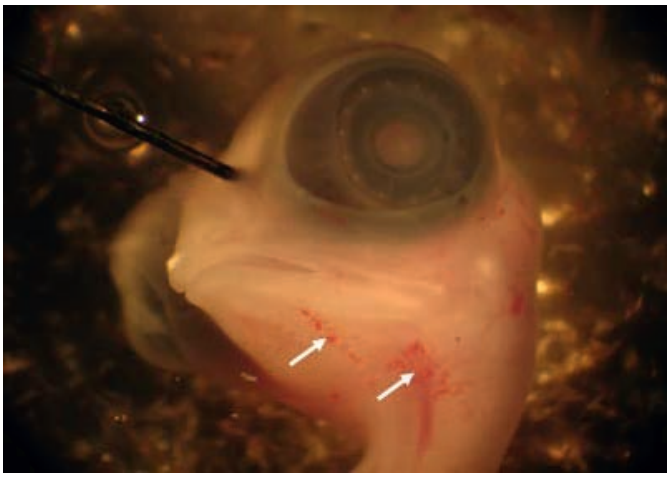

Fig. 2. Hemorrhagic spots on the head of the chick embryo after bendiocarb application

We have noted a mild increase of the cell death at 24 h but not 48 h interval (Fig. 3) revealed by whole mount staining with the vital dye, but it did not result in any congenital anomalies and was substantially smaller than the increased of cell death associated with e.g. bromodeoxyuridine embryotoxicity that does result in limb defects. Thus, we did not expect to find any significant changes in cell death patterns that were shown to be a common mechanism of pathogenesis of congenital anomalies (Sedmera and Novotna, 1994 and references therein).

Organ toxicity

The microscopic findings in CNS in chicken embryos exposed to bendiocarb at 3 ED (early developmental stage) and 10 ED (later developmental stage) were negative when compared to the control. Part of the neck was sampled for this examination (including spinal cord cross section) and no histological changes were observed in CNS as far as neurons and intracellular space was concerned (Fig. 4). Our experiment showed that application of bendiocarb to chicken embryos produced no macroscopic or microscopic changes in the CNS tissue in comparison with the control. There were no changes in the CNS tissue when bendiocarb was administered (500 μg/egg) at 3 ED, nor were these changes after application of bendiocarb (800 μg/egg) at 10 ED. When we used the highest possible dose of bendiocarb (1600 μg/egg), and at the early stage (3 ED) survived only three embryos thus the chick embryos compensated bendiocarb dose or they died. At the later stage (10 ED) the chick embryos survived while mainly thin and more sensitive individuals died.

Caspase activity

Chicken embryos were administered bendiocarb at 3 ED at doses of 500 μg/egg. Among them, we observed 450 nerve cells (with the mean number of one nerve cell/2 μm^3, in the viewing field of size 887.5 μm^3. One cell (0.20%) showed caspase activity in comparison with the control. In chicken embryos which were administered bendiocarb at 10 ED at doses of 800 μg/egg, one cell (0.20%) with caspase activity was found in comparison with the control which contained three (0.7%) red-stained nerve cells. In chicken embryos that were exposed to bendiocarb at 3 ED and 10 ED low caspase activity was detected in comparison with the control. The presence of apoptotic cells in CNS after exposure to bendiocarb can be related to physiological elimination of excessive neurons at generation of synapses (Fig. 5). In chick embryos that were exposed to bendiocarb (1600 μg/egg) on 3 ED and 10 ED were detected low caspase activity in comparison with the control.

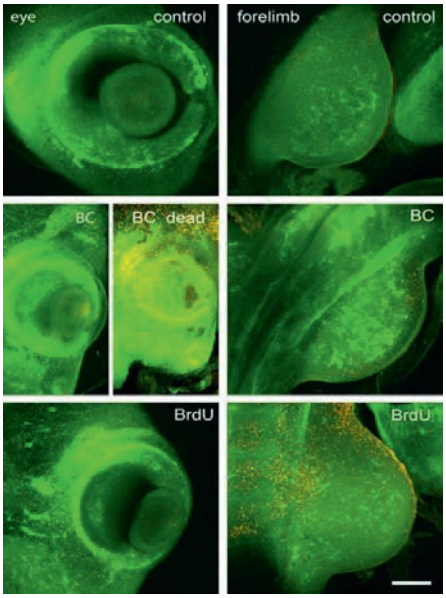

Fig. 3. Lysotracker Red (LTR) staining of ED 4 embryos in control eye and wing bud 24 hours after administration of bendiocarb (BC). A mild increase in the number of dead cells (red or yellow colored, dotted areas) is observed in the treated group, but a much more pronounced effect is visible in those treated with a teratogenic dose of BrdU (positive control). Scale bar 100 μm

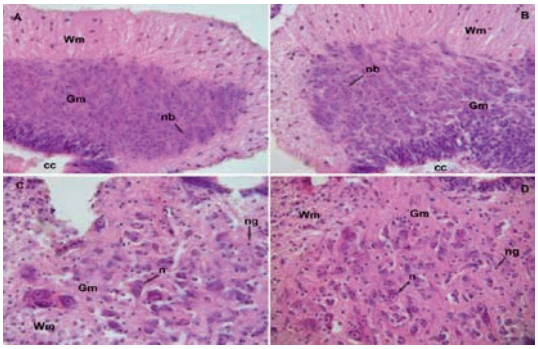

Fig. 4. Toxic action of BC on CNS of chicken embryos exposed on 3 ED (9 ED – A: control embryo, B: treatment embryo; 500 μg/egg) and 10 ED (17 ED – C: control embryo, D: treatment embryo; 800 μg/egg). White matter (Wm); gray matter (Gm); central canal (cc); neuroblast (nb); neuron (n); neuroglia (ng) [H-E, 60×]

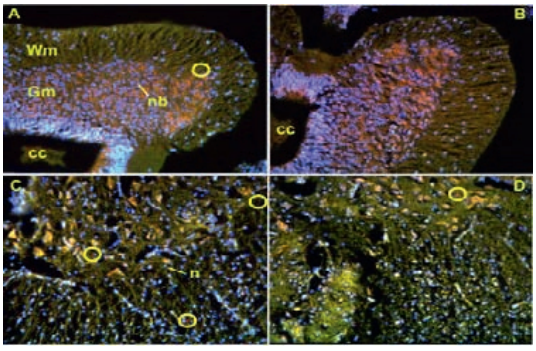

Fig. 5. Caspase activity of nervous cells (in white circle) after application of BC on 3 ED (9 ED – A: control, B: treatment embryo; 500 μg/egg) and 10 ED (17 ED – C: control, D: treatment embryo; 800 μg/egg). White matter (Wm); gray matter (Gm); central canal (cc); neuroblast (nb); nervous cell (n) [stained immunohistochemically, 40×]

Discussion

Embryotoxicity

This study provides the detailed analysis of bendiocarb toxicity in the chick embryo. Acute oral toxicity of bendiocarb has been investigated in adult mammals such as rat, guinea pig and rabbit, as well as the LD_{50} in non-mammalian species (e.g. mallard duck 3.1 mg kg^{-1}, bobwhile quail 16 mg kg^{-1}, hen 137 mg kg^{-1}; WHO, 2007), and fish 0.7–1.8 mg l^{-1} (LC_{50}; Hayes and Lawes, 1990). The wing and leg buds are similar to one another morphologically, but by about ED 4 they have

begun to acquire their individual characteristics. Hirata and Hall (2000) concluded that cell death is a feature of development. The programmed cell death plays an important role in shaping and patterning of organs during morphogenesis and organogenesis on ED 1–9 (Bellairs and Osmond, 2005).

There were no specific malformations associated with bendiocarb exposure in our set of experiments. Those encountered were also seen in the controls, and the frequency did not exceed 2%, which is considered background noise in the pre-hatching chicks (Novotna et al., 1994). While embryonic mortality is clearly correlated to the size of the dose, the number of malformed embryos does not change very much as the dose increases and may even decline (Peterka et al., 1986). Nevertheless, overriding differences in biotransformation in the fetus is the probable role of maternal metabolism of xenobiotics affecting the level of fetal toxicant exposure (Garry, 2004). It was shown in the 1930s using farm animals that embryos born to vitamin A-deficient females had many congenital defects, including abnormalities of the central nervous system (CNS) and neural crest derivatives. Conversely, it was subsequently shown in the 1950s that too much vitamin A is equally harmful to the embryo and that a similar spectrum of defects arises in the CNS and neural crest derivatives. Therefore, the correct level of vitamin A is required for appropriate CNS development and that either too much or too little is equally harmful to the embryo (Maden et al., 1998).

Cell death detected in the developing embryo could be the most sensitive indicator of toxic effects of a substance, even if they are compensated later on by increased proliferation of the remaining cells and thus fail to translate into overt malformations (Novotna and Jelinek, 1990). We noted a mild increase in the number of dead cells revealed by whole-mount staining with the vital dye, but it did not result in any congenital anomalies and was substantially smaller than the increased of cell death associated with, for example, BrdU embryotoxicity, which does result in limb defects (Sedmera and Novotna, 1994). It is possible that this mild reduction in cell number could underlie the small dose-dependent decrease in embryonic weights observed at the time of autopsy. Bromodeoxyuridine incorporation into DNA induces a dose-dependent cytotoxic effect (Fränz and Kleinebrecht, 1982). The rate of cell death in consequence of BrdU-induced DNA single strand breaks (Novotna et al., 1994) must undoubtedly influence the pattern of programmed cell death in embryonic development as well as the resulting spectrum of malformations (Sedmera and Novotna, 1994). The lack of excessive cell death in the bendiocarb group could be in consequence of less DNA damage. Subsequently, the number of malformations was low in survivors. The next bendiocarb effect could be an influence on the other cell structures and processes which cause death of the chick embryo. Toxicity to central nervous system could be another manifestation of deleterious effects of bendiocarb in the chick embryo. Histological examination of this structure did not show any significant morphological or caspase immunopositivity.

Organ toxicity

Our analysis of bendiocarb embryotoxic potential in the chick embryo supports the earlier observations in other animal models, testifying to the relative safety of bendiocarb for the embryo or fetus. A two-year study on dogs which received bendiocarb in food, revealed no changes in the weight of organs or any harmful effect of the pesticide on dog tissues. The daily dose used corresponded to 12.5 mg/kg BW and the authors detected increased serum cholesterol and decreased bloodstream level of calcium (Baron, 1991).

Toxicity of bendiocarb to organs was investigated in adult rabbits which received bendiocarb *per os* at a dose of 5 mg/kg/day. In this study, based on long-term (90 days) application of bendiocarb, the authors observed increased volume of cortex and decreased volume of thymus pulp. In addition to that, the morphometric analysis detected lower number of cells and also smaller diameter of cells in the thymus in comparison with the control (Flesarova et al., 2007). Male rats showed a significant increase in incidence of nuclear cataract related to bendiocarb dose (20 and 200 mg/kg) (Hunter et al., 2008).

Caspase activity

The presence of apoptotic cells in the liver after application of bendiocarb may be related to physiological apoptosis occurring during embryogenesis (Petrovova et al., 2009). Apoptosis is also known as "programmed cell death" because in many cases the patches of cells die in a particular location of the embryo at a specific time in development and play an important role in morphogenesis (Bellairs, 1961). Caspase-3 is a member of the family of cysteine proteases. An apoptotic signal such as granzyme B of cytotoxic T-cells induces the intracellular clevage of Caspase-3 from the inactive proform to the active form. The active form of Caspase-3 cleaves several other apoptotic proteins (Fernandes-Alnemri et al., 1994). The experiment based on application of bendiocarb to chicken embryos at 3 ED and 10 ED showed no increase in the number of CNS cells with caspase activity in comparison with the control. Cell death with its well-known role in morphogenesis is an important characteristic of developing legs in chicken embryos (Dawd and Hinchliffe, 1971). During the development of limbs the cell death results in removal of interdigital tissue and in birds also to vanishing of the 1st and 5th toe. In this way the cell death participates in formation and development of toes of bird legs. Cell apoptosis is species-specific not only from temporal but also from the spatial point of view (Zakeri and Lockshin, 2002).

Cell apoptosis occurs in chicken embryos for the first time at 2 ED (somites and neural tube). The interdigital regions of mesenchyma are subject to regression and in this way they are likely to participate in formation of toes in amniotic em-

bryos (chicken embryo, murine embryo and others) and also in humans (Dawd and Hinchliffe, 1971). Cell apoptosis has an important role also in the nervous system. In the course of development of vertebrates the nerve cells are produced in excessive numbers and therefore cellular apoptosis involving 20–80% of neurons is physiological. Fetal neurons thus compete for *nerve growth factor* (NGF) which ensures their survival and is produced not only by neurons but also by other cells. However, not all cells obtain the required quantity of NGF for their survival. Therefore apoptosis adjusts the total number of produced neurons to such quantity which is supported physiologically (Zakeri and Lockshin, 2002).

Conclusions

Adverse health effects of pesticides in humans cover a variety of domains; some compounds may only exert some mild irritant in the skin, while others may affect the organs (liver, kidneys or lung functions). Some of them are carcinogenic, and other may cause reproductive toxicity or have endocrine disrupting properties. Many pesticides target the nervous system of insect pests. Because of the similarity of neurochemical processes, these compounds are also likely to be neurotoxic to human. *In vitro* systems such as neural cell lines or embryo cultures can play key roles in elaborating of the effects of prenatal exposure to cholinergic pesticides and in establishing new safety thresholds for insecticide exposure during development. Carbamate insecticides have different degrees of acute oral toxicity, ranging from moderate to low toxicity (carbaryl – 250 mg/kg), to extremly high toxicity (aldicarb – 0,8 mg/kg) (Lotti and Moretto, 2006). In comparison with classic methods of toxicological study, the chick embryo technique is unique with respect to mechanism of exposure of test organism to xenobiotics. We conclude that bendiocarb does not possess a significant neurotoxic potential, at least in the avian embryo, but this result cannot be directly extrapolated to the mammals. Large doses would impair maternal metabolism and could cause secondary problems to the developing embryo or fetus in mammals. Nevertheless, the results gained on the chick embryos could be predicted as potential biomarkers of pesticide toxicity. Further studies on laboratory animals (mouse, rat, rabbit) will have to be conducted for better understanding of developmental neurotoxicity of bendiocarb.

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