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Morphogenetic Activities of Bendiocarb as Cholinesterase Inhibitor on Development of the Chick Embryo

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1. Introduction

1.1 Xenobiotics and pesticides

Xenobiotics are exogenous foreign substances with different effects on living organism. One group of xenobiotics present agrochemicals including pesticides that can be related to many health disorders. Besides acute intoxication these can also cause motor and sensory disorders (e. g. Parkinson's disease), carcinogenic diseases, reproduction disorders, immunosuppression and allergic states, etc. From a medical view these exogenous foreign environmental compounds commend great respect. The animal eukaryotic multicellular organism is a complicated multistage process at the molecular, physiological and morphological levels. Various negative regulating factors can pass into their processes. Teratogenesis and consequently malformation, eventually death of embryo or foetus can be a result. Some pesticide action of cholinesterase inhibitors, which prevent of inhibit cholinesterase from cleaning up unused acetylcholine also belong to this group. Many of these xenobiotics are certified or assumed to be carcinogenic, embryotoxic or foetotoxic and teratogenic chemical compounds.

Agrochemicals, as chemicals used in agriculture, include a broad range of products used for the nutrition of plants, protection of fruits, protection and nutrition of animals. There are pesticides, substances used as repellents or destroyers of all sorts of plant and animal pests. At present, among more than 700 registered pesticides around the world, there are herbicides, insecticides, fungicides, rodenticides, nematocides, bactericides, algicides, molluscocides, ascaricides, etc. They are exogenous foreign substances, xenobiotics, with various consequences for living organisms. Their effect depends mainly from substances activity, from their metabolizing, respectively (Wylie et al., 2005).

It is known that pesticides can cause acute intoxication or carcinomas. Many of these suppress the immune system which increases its susceptibility to virus, bacteria and parasitic infections and to tumours. It is known that farmers are population with a high risk of Hodgkin's disease, melanomas, multiply myeloma and leukaemia.

Many agrochemicals (xenobiotics) including pesticides, in the liver through detoxication can be transformed to reactive compounds with mutagenic, carcinogenic or teratogenic metabolites (so-called biotransformation).

Many pesticides, mainly insecticides are introduced among cholinesterase inhibitors, which commonly have an intimate relation to animal morphogenesis. Pesticides are a group of chemicals with high biological activity that are worldwide introduced into the environment and expose large populations of living organisms. Pesticides possess properties that make them different from other chemicals mainly because they are introduced to environment. They are important for their stability in the environment, exposure of population and high biological activity. Though, toxic effect of the pesticides is specialized to specific species. They may endanger also human health, and both domestic and wildlife animals. The annual application of synthetic pesticides to food crops in the EU exceeds 140,000 tones, an amount that corresponds to 280 grams per EU citizen per year. Many pesticides, mainly insecticides are introduced among cholinesterase inhibitors, which commonly have an intimate relation to animal morphogenesis.

1.2 Morphogenetic roles of acetylcholine and cholinesterase

The biological process of morphogenesis is a process in which living systems produce forms and structures through mechanical and biological factors including morphogens. These are developmental signals that exert specific effects on receptive cells depending on their concentration. In developing tissues, neurotransmitters subserve growth regulatory and morphogenetic functions. The stimulation of cholinergic receptors in target cells during a critical developmental period provides signals that influence cell replication and differentiation. Accordingly, environmental agents that promote cholinergic activity evoke neurodevelopmental damage because of the inappropriate timing or intensity of stimulation.

Morphogens are developmental signals that exert specific effects on receptive cells, depending on concentration. Morphogens are present in gradients created by the presence of a "source" and "sink". Developing cells are affected in specific ways along this concentration gradient. This concept has traditionally been applied to substances involved in pattern formation and morphogenesis, such as retinoic acid. However, it may also be appropriate to consider neurotransmitters as morphogens when they act as dose-dependent morphogenetic signals in neural and non-neural tissues (Lauder, 1988 as cited in Lauder & Schambra, 1999).

Neurotransmitters participate in various forms of intra- and intercellular signaling throughout all stages of ontogenesis, (Buznikov et al., 1996) and they exert their effects using receptors and signal transduction mechanisms similar to those in the adult nervous system (Lauder, 1985, as cited in Slotkin, 1999). These substances and their specific receptors has been identified during ontogeny of the mammalian nervous system, and it is now certain that transmitters play essential roles in the cellular and architectural development of the brain (Whitaker-Azmitia, 1984, as cited in Slotkin, 1999). During this period, receptor stimulation uniquely communicates with the genes that control cell differentiation, changing the ultimate fate of the cell. The ontogenetic state of the target cell is critical in determining whether the outcome of receptor stimulation is an effect on cell replication, differentiation, growth, death (apoptosis), or "learning," that is, determining the future set-point for responsiveness of the cell. At the same time, these multiple roles create a wide

window of vulnerability in which exposure of the brain to neuroactive chemicals that elicit or block neurotransmitter responses can alter development (Yanai, 1984, as cited in Slotkin, 1999). Thus, unlike classical teratology, in which 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).

Acetylcholine (ACh) is synthesized from acetyl coenzyme A and choline by the enzyme choline acetyltransferase. In addition to its synthesis in the liver, choline employed in acetylcholine production is derived from dietary sources. There is a carrier system in capillary endothelial cells that is responsible for transport of choline, in its free and phospholipid forms, into the brain.

ACh is a neurotransmitter widely diffused in central, peripheral, autonomic and enteric nervous system. 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). Acetylcholine is a major excitatory neurotransmitter in the nervous system of vertebrates and invertebrates. Accumulated evidence suggests that ACh also 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). Evidence that ACh plays a key role in neural development suggests that disruptors of cholinergic function could disturb these actions if present during key critical periods. The cerebral cortex may be especially vulnerable to such insults because of important roles cholinergic afferents play in cerebral morphogenesis and synaptogenesis. Disruptors of cholinergic function that may have significant effects on brain development include alcohol, nicotine, and cholinergic pesticides (Slotkin, 1999).

In sea urchin embryos, cell movements occurring during gastrulation and postgastrulation stages appear to be regulated by ACh and biogenic monoamines (Falugi, 1993). Similar functions of ACh during gastrulation of vertebrate embryos are suggested by the presence of AChE during gastrulation in the chick embryo (Laasberg et al., 1987 as cited in 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. New evidence that AChE may have a direct role in neuronal differentiation provides additional grounds for interest in the developmental toxicity of anticholinesterases. Still, developing rats recover faster from AChE inhibition than adults, largely due to the fact that developing organisms have a rapid synthesis of new AChE molecules. It therefore seems that either developmental toxicity may unrelated to AChE inhibition, or that even a brief period of AChE inhibition is sufficient to disrupt development (Slotkin, 2004). Some selective cholinesterase inhibitors effectively suppress neurite outgrowth in model systems like differentiating neuroblastoma cells and explanted

sensory ganglia. Certain of these "morphogenic" effects may depend on protein-protein interactions rather than catalytic AChE activity. It remains possible that some pesticides interfere with important developmental functions of the cholinesterase enzyme family (Brimijoin & Koenigsberger, 1999). Insecticides which enhance cholinergic effects through inhibition of cholinesterase are the most widespread chemical assaults on the fetus.

AChE is the enzyme that hydrolyzes the neurotransmitter acetylcholine at cholinergic synapses and neuromuscular junctions. The reaction that is catalysed by AChE is: $\text{acetylcholine} + \text{H}_2\text{O} \rightarrow \text{choline} + \text{acetate}$. AChE is present in mammals, birds, fish, reptiles and insects (Fukuto, 1990, as cited in Van Dyk & Pletschke, 2011). AChE predominates in neurons and muscle cells wherever cholinergic synapses are found. But the full picture is more complex, since AChE also occurs in nonneural and embryonic tissues like red blood cells, megakaryocytes, and migrating neural crest cells (Brimijoin & Koenigsberger, 1999). Proposed noncholinergic roles for AChE range from neuromodulation by secreted forms to promotion of cell proliferation in tumor growth and hematopoiesis (Soreq et al., 1994).

Prenatal exposure to organophosphate and carbamate pesticides could have adverse effects on neural development by interfering with the morphogenic function of AChE. Accumulating evidence indicates that AChE has extrasynaptic functions during neural development (Layer & Willbold, 1995, as cited in 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. Together, these data strongly suggest that AChE plays a developmental role in the morphogenesis of the nervous system (Bigbee et al., 1999).

Although AChE may affect morphogenesis by noncatalytic mechanisms such as structural recognition, these mechanisms could certainly be vulnerable to pesticides. However, the toxicologic data reviewed earlier indicate that those agents may have additional actions that would be deleterious to a growing nervous system. It seems wise to re-evaluate the developmental risks of anticholinesterases as data become available from ongoing studies of environmentally relevant molecules in neuronal culture and sensitive embryologic models of neural development (Brimijoin & Koenigsberger, 1999).

Today we know three isoforms of AChE:

- AChE - S (synaptic soluble) the major multimeric enzyme of brain and muscles,
- AChE - R ('readthrough') monomeric enzyme of embryonic and tumor cells
- AChE - E (erythrocytes) associated with erythrocyte membranes.

Prenatal development of the central cholinergic nervous system coupled with the important developmental roles played by ACh in both neural and non-neural tissues should make the vertebrate embryo especially vulnerable to the gestational effects of environmental neurotoxins that target cholinergic receptors or choline esterase. It seems especially important to study of effects of chronic prenatal exposure to cholinergic pesticides on pre- and postnatal brain development as well as behavioral consequences of these exposures (Lauder & Schambra, 1999). Drugs or chemicals that target cholinergic neurotransmission probably represent the largest source of neurobehavioral teratogenesis. Agricultural and

household pesticides that target AChE could interfere with non-cholinergic role of AChE if exposure occurs during critical periods of nervous system development (Bigbee et al., 1999). Given the widespread use and exposure to pesticides, the general lack of data on developmental neurotoxicity is a serious impediment. For certain pesticides, a requirement exists for neurotoxicity tests in adult animals, but developmental neurotoxicity is usually not considered when determining pesticide safety. Experimental, clinical, and epidemiologic evidence suggests that neurotoxic pesticides can also cause developmental neurotoxicity, and that the effects are more severe and lasting, and that they occur at much lower exposure levels. Given the likely environmental etiology of neurodevelopmental deficits and their importance to families and to society, prevention of exposures to neurotoxic pesticides should be made a public health priority (Bjørning-Poulsen et al., 2008).

1.3 Carbamates and organophosphates like inhibitors of cholinesterase

Decades ago the activity of acetylcholinesterase (AChE) was used as a sensitive indicator of exposure to organophosphate and carbamate pesticides. The inhibition of AChE is associated with a toxic mechanism of pesticide, with the pesticide's reversible or irreversible binding to the ester-point of enzyme and with increasing of cholinergic effects on the nervous system (Kristoff et al., 2006). The inhibition of AChE by organophosphates takes place as a result of the phosphorylation of the serine residue in the active site of the enzyme. Carbamate pesticides are cholinesterase inhibitors with a similar mechanism of action as organophosphate pesticides. However, carbamates cause only reversible inhibition of AChE. Thus, AChE inhibition by carbamates lasts only minutes or hours, whereas the effects of organophosphates (OPs) with respect to AChE can last for 3–4 months (Van Dyk & Pletschke, 2011). A number of pesticides belong to the group called cholinesterase inhibitors (ChEI), which not only in one case report the relation to morphogenesis. ChEI form a group of chemical compounds that prevent hydrolysis of acetylcholine (ACh; as a classical neurotransmitter of the autonomic nervous system providing communication between cells), thereby allowing accumulation of ACh in the reactive sites of the living organism. Such activity may have some drugs (eg. Alzheimer's disease and other dementia diseases called memory drugs) but also insecticides, respectively some chemical warfare agents that can be fatal for humans and animals (Krall et al., 1999).

Due to the fact that AChE is inhibited, ACh is not hydrolyzed and its accumulation occurs on the receptors of target cells. Cumulation effects are manifested by excessive stimulation of cholinergic synapses in the central and peripheral nervous system (Huff et al., 1989, Ratner et al. 1983). The most common accompanying symptom is decreasing of AChE activity in blood and dysregulation of ions between the external and internal environment causing failure of formation of action potentials in nerve endings (Kassa & Samnaliev, 2004; Mignini et al., 2003).

Changes induced by the action of ChEI can be divided into two effects:

1. muscarinic effect and
2. nicotine effect.

Both have central and peripheral effects.

Peripheral nicotinic effects manifest on the periphery causing fasciculation, muscle contraction, muscle pain, general weakness, tachycardia, hypertension, hyperglycaemia and mydriasis.

Peripheral muscarinic effects in the periphery cause contraction of smooth muscles, stimulation of the glands, increased salivation, lacrimation, rhinorrhoea, bronchial secretion

(bronchorea) bronchial constriction, cyanosis, nausea, vomiting, incontinence (incontinentio urinae), bradycardia and pulmonary edema.

Central effect causes insomnia, sleep disorders, headaches, dizziness, behavioral disorders, tremor, ataxia, respiratory depression, convulsions and coma (Levelridge, 1998).

1.3.1 Organophosphates

OPs belong to a group of insecticides, which have been discovered in 1938 by German chemists. OPs were used as nerve poisons during the 2nd World War. At present, the OPs are used in agriculture and also used as antiparasitic substances for destroying insects such as fleas, louse and mosquitoes.

OPs replace forbidden organic chlorine compounds and they are a major cause of poisoning in animals. They vary in toxicity, the level of residue and excretion. Many OP were developed to protect plants and animals and generally have an advantage by creating little or no residues in tissues and environment. But it seems that chlorinated OP compounds have greater potential for producing tissue residues. Many OPs used as pesticides are not strong inhibitors of esterases, until they are activated in the liver by microsomal enzymes (Toxicology, 1998).

In severe cases of OP poisoning in adults (AChE inhibition exceeding 70%), a “cholinergic syndrome” is elicited, including various central nervous system effects such as headache, drowsiness, dizziness, confusion, blurred vision, slurred speech, ataxia, coma, convulsions and block of respiratory centre. Some OPs can also induce a delay neuropathy which does not involve inhibition of AChE but rather the neuropathy target esterase (NTE). The physiological functions of NTE are still unknown, and it is obscure how phosphorylation and aging of NTE leads to axonal degeneration. The syndromes described above are observed only following high dose, acute exposures to OPs. Survivors recover from these syndromes, but it is likely that the exposure also produces long-term adverse health effects. In rats, a single high exposure to an OP can cause long lasting behavioural effects and the same has been reported from several human studies (Bjørning-Poulsen et al., 2008). WHO estimates that each year there are 3 million cases of acute pesticide poisoning, and it ends with 220 000 deaths (Jaga & Dharmani, 2003).

1.3.2 Carbamates

Carbamates (CAs) are substances that were originally extracted from the bean called calabar, which is the home plant of Western African States. The obtained extract contained esters of physostigmine and methylcarbamate. CA is considered a derivative of carbamic acid. CAs act as acetyl cholinesterase (AChE) inhibitors that affect lots of organs such as peripheral and central nervous systems, muscles, liver, pancreas, and brain. There are several reports about metabolic disorders, hyperglycemia, and also oxidative stress in acute and chronic exposures to pesticides that are linked with diabetes and other metabolic disorders. Induction of oxidative stress by some carbamates might also cause developmental neurotoxicity. In this respect, there are several in vitro and in vivo but few clinical studies about mechanism underlying these effects (Karami-Mohajeri & Abdollahi, 2010). When comparing the clinical course of carbamate poisoning (by aldicarb or methomyl) in young children (1–8 years old) and adults (17–41 years old), it was found that the predominant symptoms in children were CNS depression and hypotonia, and the most common muscarinic effect was diarrhoea. In adults the main symptoms were miosis and

fasciculations, whereas CNS depression, hypotonia, and diarrhoea were uncommon. As for the OPs, it is likely that poisoning with carbamates may result in long term neurological effects. Two patients showed cognitive deficit in attention, memory, perceptual, and motor domains 12 months after a poisoning incident. With respect to long term, low level exposures to carbamates, reports concerning chronic toxicity are almost non-existent. (Bjørning-Poulsen et al., 2008). Results indicated that CAs impair the enzymatic pathways involved in metabolism of carbohydrates, fats and protein within cytoplasm, mitochondria, and proxisomes (Karami-Mohajeri & Abdollahi, 2010). Also, carbamate insecticides inhibit cellular metabolism including energy, protein, and nucleic acid metabolism, thereby, causing cell regression and death (Mohd.Amanullah & Hari, 2011).

Carbamates represent except AChE inhibitors also inhibitors of brain esterase (NTE neuropathy target esterase) leading to polyneuropathy. This neuropathy arises due to degeneration of long axons of nerve cells. Brain NTE esterase is a protein that is present in neurons as well as in other vertebrate cells and plays a role in the interaction between neurons and glial cells relevant in the evolving nervous system (Glynn, 1999). Recent studies suggest that carbamates cause virtually 100% inhibition of NTE and polyneuropathy in chicken models (Lotti & Moretto, 2006).

No epidemiological studies of developmental neurotoxicity of carbamates in humans could be found, and data from animal experiments are very sparse as well. Assuming that some of the neurotoxic effects observed in association with prenatal exposure to OPs, such as chlorpyrifos, are due to inhibition of AChE, it is possible that carbamates may have similar developmental effects, even though the inhibition of AChE by carbamates is only transient (Bjørning-Poulsen et al., 2008).

1.4 Bendiocarb

Bendiocarb (2,3-isopropyledene-dioxyphenyl methylcarbamate) is a carbamate insecticide, which also belongs among the ChEIs, and is used to control disease vectors such as mosquitoes and flies, as well as household and agricultural pests. Most formulations of bendiocarb are registered for general use, except to Turcam, Turcam 2.5 G and the best-known product Ficam (Flesarova et al., 2007). The blockage of enzyme cholinesterase (ChE) caused by bendiocarb persists for approximately 24 hours and subsequently the situation returns to normal after acute exposure because the insecticide does not accumulate in mammalian tissues (Sirotakova et al., 2005). Bendiocarb is the pesticide acting upon invertebrates by irreversibly blocking the activity of the ChE, which is critical in allowing muscle relaxation by removing the neuromuscular mediator ACh. Acute toxic symptoms of carbamate poisoning, e.g. miosis, urination, diarrhea, diaphoresis, lacrimation, salivation, and excitation of the central nervous system, are generally caused by inhibition of the AChE, which leads to accumulation of ACh. An acute oral toxicity (LD_{50}) was investigated in different adult mammals; rat 34-156 mg kg^{-1} , guinea pig 35 mg kg^{-1} , rabbit 35-40 mg kg^{-1} ; and also in non-mammalian species like birds: mallard duck 3.1 mg kg^{-1} , bobwhite quail 16 mg kg^{-1} , hen 137 mg kg^{-1} (World Health Organization [WHO], 2007), fish 0.7 - 1.8 mg kg^{-1} (LC_{50} , Hayes & Lawes, 1990); bee 0.1 μg per bee (Wright et al., 1981).

Studies on chronic exposure to carbamate insecticides and case reports of long-term exposure give equivocal results. Chronic intoxication with bendiocarb was investigated in 2-year study on adult rats that administered bendiocarb orally in a dose of 10 mg/kg/day. The author observed changes in the weight of organs, composition of blood and urine and

also increased occurrence of stomach and eye lesions (Baron, 1991). Adult rabbits that were administered bendiocarb per os for 90 days at the dose 5 mg/kg/day, showed slight toxic effect of bendiocarb. However, no negative effect of bendiocarb was observed on formation of thymus structures (Flesarova et al., 2007). In a three-generation reproductive study in rats, the no-effect levels of bendiocarb administered in the diet were considered to be 0.6 and 3 mg kg⁻¹ body weight per day for reproductive effects respectively. No teratogenic effects were seen in the offspring of rats given 4 mg kg⁻¹ or in rabbits given 5 mg kg⁻¹ per day of bendiocarb during gestation (Kamrin, 1997). Up to now, in the organotoxic (acute and chronic toxicity) and ecotoxic properties, moreover reproductive, teratogenic, mutagenic and carcinogenic effects of this insecticide have been studied. No epidemiological studies of developmental neurotoxicity of carbamates in humans could be found, and data from animal experiments are very sparse as well.

Currently the oral LD₅₀ of bendiocarb for hen is 137 mg/kg.b.w (WHO, 2007). Up until now no detailed studies were conducted regarding the embryotoxic effects of bendiocarb on birds which are more sensitive to the action of toxic substance.

1.5 Animal model - chick embryo

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. 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.

Chick embryo is a popular model for developmental pharmacological and toxicological studies. It is readily available, cost-efficient, and presents an alternative approach to treatment of pregnant mammals. The concordance of data from CHEST 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. 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. The presumptive liver areas of the chick embryo are closely associated with those of the heart and together are known as the cardio-hepatic regions. Whereas the heart is an entirely mesodermal structure, the liver is formed from both, mesoderm and endoderm. The liver primordium is visible at the end of 2 ED. As it grows, it comes into contact with the body wall (Bellairs & Osmond, 2005).

In order to assess to the fullest extent the possible embryotoxic potential, we performed a detailed study of bendiocarb effects in the chick embryo.

We observed the toxicity (mortality and weight of survived embryos - LD₅₀) of bendiocarb and the associated occurrence of malformations during various developmental stages (embryonic days 2-5 and 10). Then we observed the organ toxicity as well as the programmed cell death (apoptosis) after bendiocarb administration. Bendiocarb was administered to individual developmental stages in various doses (8-1600 µg/egg).

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 pesticides residues directly affect the embryos, disturbing their normal development and causing pathophysiological and morphological changes. The aim of our study was to investigate toxicity of cholinesterase inhibitor bendiocarb to organs (liver, CNS) of the chick embryo and also the entire embryotoxicity in the chick embryo.

2. Material and methods

2.1 Eggs

Fertilized white Leghorn chicken eggs were purchased from the animal facility of the Institute of Molecular Genetics (Koleč, Czech Republic) and 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. Embryos were observed during incubation and dead, growth retarded or dysmorphic individuals at the time of treatment were excluded from further study.

2.2 Bendiocarb

The bendiocarb (2,2-dimethyl-1,3-benzodiol-4-yl-N-methyl carbamate, Bendiocarb tech, 98.9%, Bayer, Germany) was dissolved in acetone and diluted with sterile water intended for tissue culture to obtain the required concentrations.

2.2.1 Application of bendiocarb

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. The tested solution was applied directly over the embryo on the top of inner shell membrane (*membrane papyracea*). 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.

2.2.2 Application dose

The application dose per one egg was 200 µL, with acetone concentration equal to 10 µL/200 µL of application dose.

2.3 Processing of the chick embryo

At the time of bendiocarb application 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 chick embryos were removed from the eggs using a crook, weighed and examined under a dissecting microscope for external (eye, beak, palate, body wall, limbs) and internal (gastrointestinal system, microdissection of the heart) anomalies.

	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
	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

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

2.4 Methods

2.4.1 Light microscopy – organ toxicity - general microscopic changes

After the examination under the dissecting microscope the embryos were fixed for 24 hours in Dents' solution (20% dimethyl sulphoxide and 80% methanol) and processed by a standard way for histological examination. Neck and part of the liver were separated from the fixed chicken embryos (exposure at 3 ED and 10 ED, right liver lobe from embryos

exposed at 10 ED). 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 liver and 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 60 x magnification. Pictures were taken subsequently using a digital camera DP 70 and Cell P (Olympus) software.

2.4.2 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 liver and 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 liver, the 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 60 x magnification.

2.4.3 Confocal microscopy – embryotoxicity - detection of 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 & Novotna, 1994). Images acquired in the green and red channels on a Leica SPE confocal microscope were processed using Adobe Photoshop.

2.5 Statistical analysis

Statistical comparison of different groups was performed using the statistical software GraphPad Prism. Value of $P < 0.05$ were considered significant.

3. Results and discussion

3.1 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 embryoletality (expressed as LD₅₀; Table 2) decreased with increasing age (Fig. 1), except on ED 3, when the LD₅₀ was the lowest. The embryoletality after bendiocarb application on ED 10 could not be determined

because of solubility limits (200 g l⁻¹ of acetone, but lower in mixture with water for injection; too much of concentrated acetone is toxic to the embryo).

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	0,973
	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	
	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	0,646
	16	24	6	25	1	1,421	0,183	
	160	19	2	11	0	1,536	0,111	
	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	1,783
	16	17	1	6	0	1,162	0,151	
	160	18	1	6	0	1,196	0,119	
	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	28,571
	80	22	9	41	0	1,445	0,187	
	160	21	11	52	1	1,635	0,220	
	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	not determined
	800	17	3	18	3	14,86	2,543	
	1600	15	5	33	0	15,30	1,337	
Total		651	197		12			

* Wet weight of embryos sampled on ED10, except for application at ED10 when sampling was done at ED17.

Table 2. Bendiocarb embryotoxicity at different stage of development

Table 2 lists the wet weight of embryos in different treatment groups. 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).

P values (differences in weight) considered statistically significant are in **bold**.

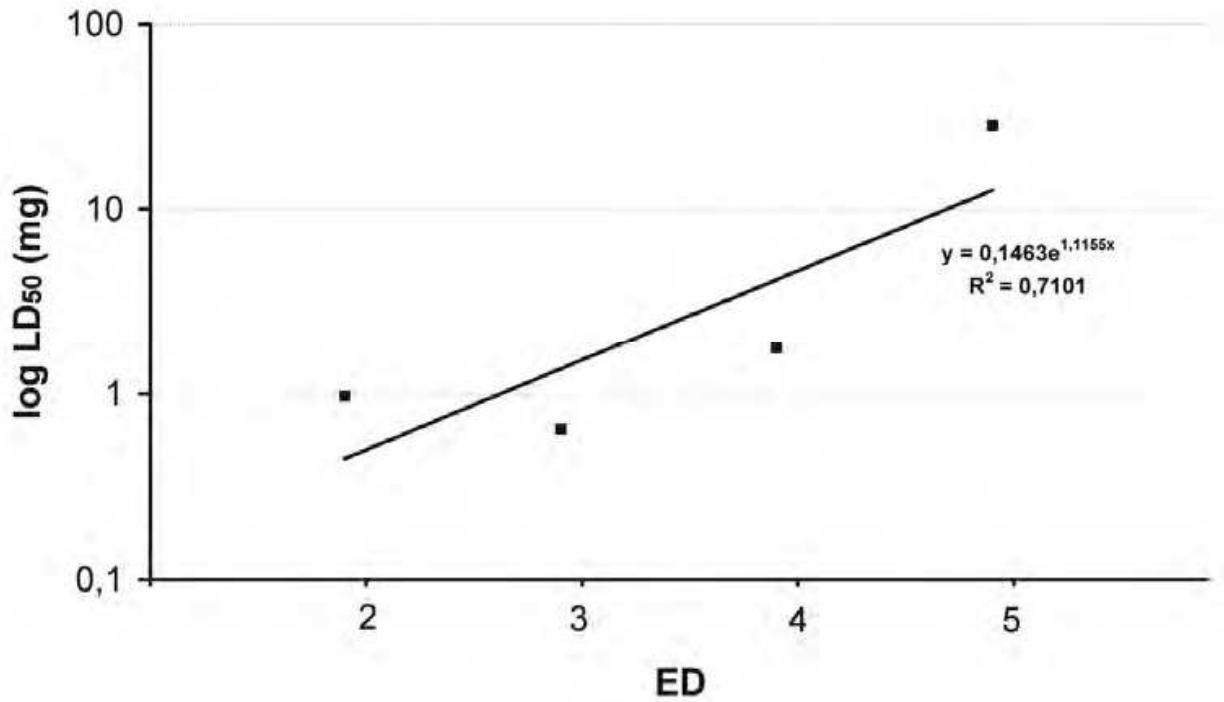


Fig. 1. LD₅₀ of bendiocarb increases with development. The graph is based on values from Table 2

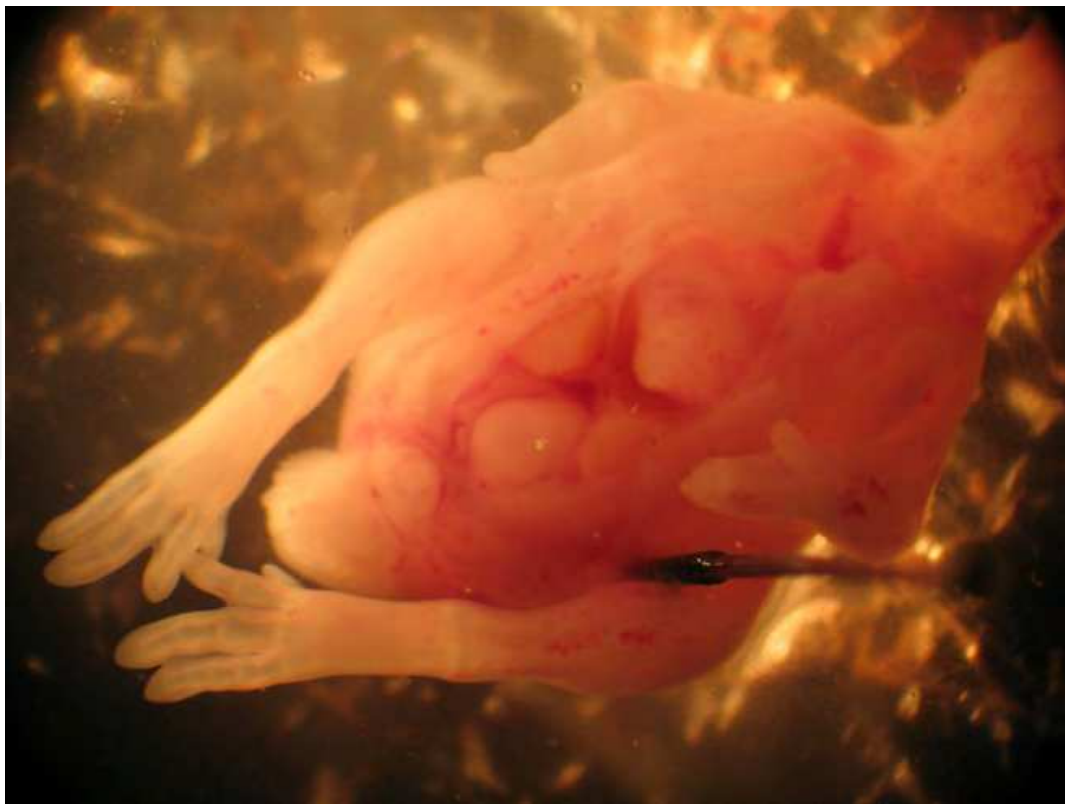


Fig. 2. Opening of the body cavity of the chick embryo

The values of mortality are used for construction of Figure 1. The weights of embryos at incubation days 2, 3, 4, 5, and 10 according to Clark et al. (1986) are 5, 18, 80, 149, and 2820 mg, respectively.

The embryoletality (LD_{50}) could not be determined on ED 10 because of solubility limits (200 g/l of acetone, but lower in mixture with water for injection).

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 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.

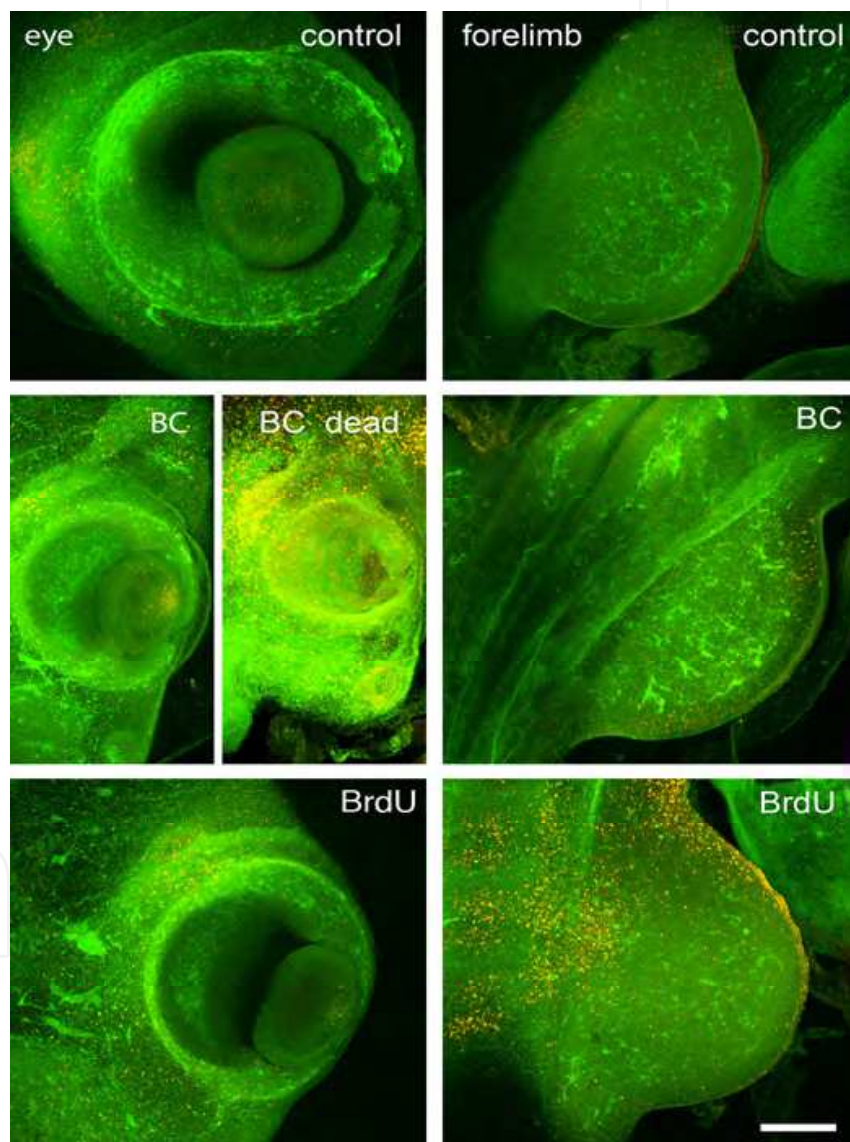


Fig. 3. Lysotracker Red (LTR) staining of ED 4 embryos in control eye and wing bud 24 hours after administration of bendiocarb (BC). A mild increase in the number of dead cells (red or yellow colored, dotted areas) is observed in the treated group, but a much more pronounced effect is visible in those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

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). 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 & 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 & Osmond, 2005). Since there was no specific pattern of malformations following bendiocarb administration, 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 & Novotna, 1994). 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 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 (Bjørning-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 (Bjørning-Poulsen et al., 2008).

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 LD₅₀ in non-mammalian species [e.g. mallard duck 3.1 mg kg⁻¹, bobwhite quail 16 mg kg⁻¹, hen 137 mg kg⁻¹ (WHO, 2007), and fish 0.7-1.8 mg l⁻¹ (LC₅₀, Hayes & Lawes, 1990)]. 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 (Table 2; calculated LD₅₀ 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.

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). We thus conclude that bendiocarb does not possess a significant teratogenic potential, at least in the avian embryo. 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). This could cause secondary problems to the developing embryo or fetus in mammals.

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 & 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, bromodeoxyuridine embryotoxicity (Sedmera & Novotna, 1994), which does result in limb defects. 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. The validity of using LysoTracker Red for whole-mount detection of cell death was verified using bromodeoxyuridine in a parallel experiment. Embryos treated with 5 µg of bromodeoxyuridine served as internal controls, and showed clearly increased amounts of cell death at 24 h but not 48 h interval (Fig. 3).

BrdU 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 (Novotná et al., 1994) must undoubtedly influence the pattern of programmed cell death in embryonic development as well as the resulting spectrum of malformations (Sedmera & 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 specific target organs such as liver and central nervous system could be another manifestation of deleterious effects of bendiocarb in the chick embryo. Histological examination of these structures did not show any significant morphological or caspase immunopositivity.

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.

3.2 Organ toxicity

At 3 ED the mortality of 51 chicken embryos used was 28% (51/14). An average weight of the control (n=36) was 1446 ± 0.165 mg. The chicken embryos exposed to bendiocarb were lower weight than control within 9% (1312 ± 0.131 mg). At 10 ED the mortality rate of 29 chicken embryos was 10% (29/3). An average weight of the control (n = 12) was 17.25 ± 1.324 g. The chicken embryos exposed to bendiocarb were lower weight than control within 14% (14.86 ± 2.544 mg).

3.2.1 Liver

Comparison with the control showed neither macroscopic nor microscopic changes in chicken embryos exposed to bendiocarb at 3 ED at concentrations of 500 µg/egg. Macroscopic observation revealed no changes in the size or shape of the liver. The organs were yellow, with a shiny surface and the sections showed preservation of characteristic liver structure. Histology of liver tissue was unchanged. We failed to observe any changes in hepatocytes or the intracellular space.

Similarly the examination of chicken embryos exposed to bendiocarb at 10 ED at doses of 800 µg/egg, failed to show any macroscopic or microscopic changes in the liver in

comparison with the control. Macroscopic examination detected no changes in the size or shape of the liver. The organ was yellow, with a shiny surface and respective sections showed that the liver structure was preserved. Histological examination of liver also failed to detect any changes as in the hepatocytes as in the intracellular space (Fig. 4).

3.2.2 Central nervous system

The microscopic findings in CNS in chicken embryos exposed to bendiocarb at 3 ED and 10 ED 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. 5).

Our experiment showed that application of bendiocarb to chicken embryos produced no macroscopic or microscopic changes in the liver and CNS tissues in comparison with the control. There were no changes in the tissues of liver and CNS when bendiocarb was administered (500 µg/egg) at 3 ED, nor were these changes after application of bendiocarb (800 µg/egg) at 10 ED.

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 b.w. and the authors detected increased serum cholesterol and decreased bloodstream level of calcium (Baron, 1991).

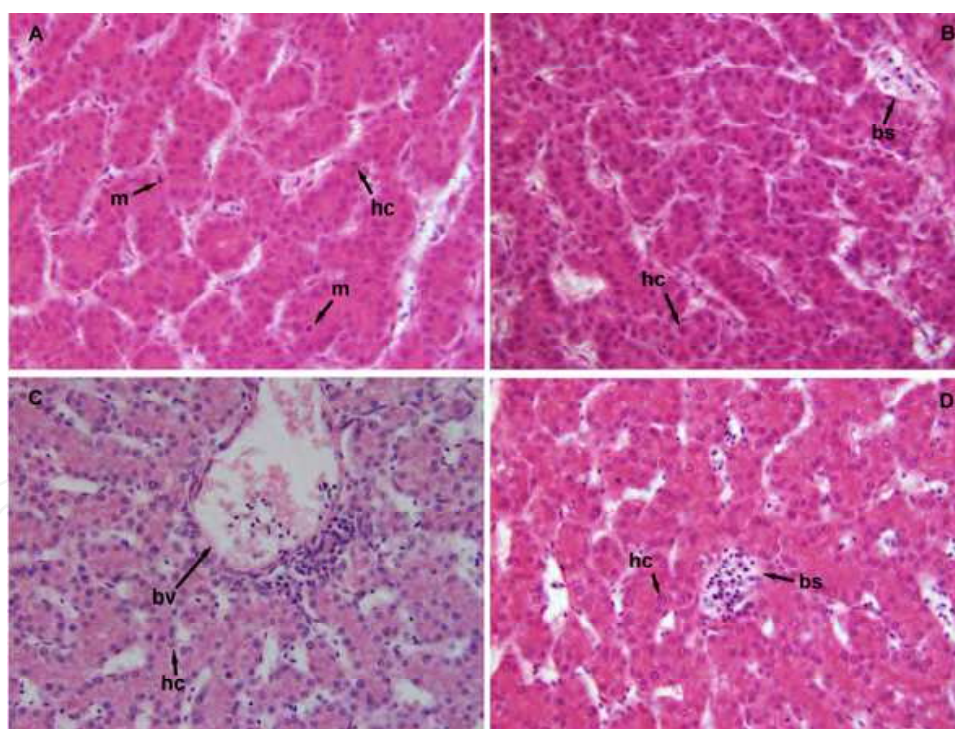


Fig. 4. Toxic action of BC on liver exposed on 3 ED (9 ED - A: control embryo, B: treatment embryo; 500 µg/egg) and 10 ED (17 ED - C: control embryo, D: treatment embryo; 800 µg/egg). mitosis (m); hepatocyte (hc); blood vessel (bv); blood sinusoid (bs) [H-E, 60x]

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).

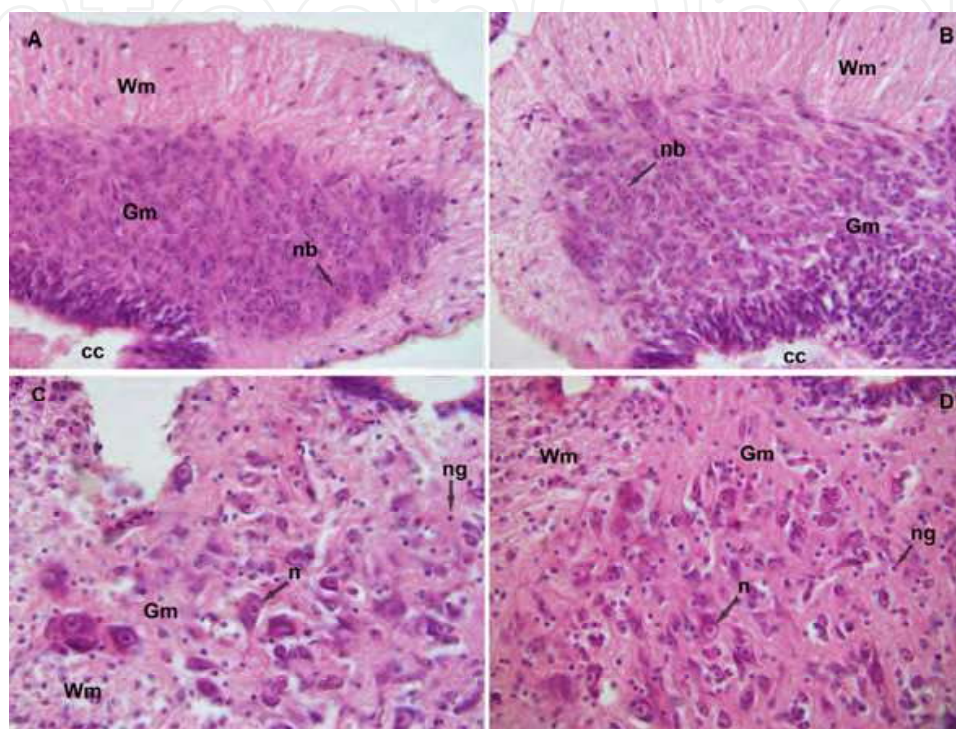


Fig. 5. Toxic action of BC on CNS of chicken embryos exposed on 3 ED (9 ED - A: control embryo, B: treatment embryo; 500 µg/egg) and 10 ED (17 ED - C: control embryo, D: treatment embryo; 800 µg/egg). white matter (Wm); gray matter (Gm); central canal (cc); neuroblast (nb); neuron (n); neuroglia (ng) [H-E, 60x]

3.3 Caspase activity

3.3.1 Liver

The chicken embryos that were exposed to bendiocarb at 3ED at concentrations of 500 µg/egg, showed low caspases activity in comparison with the control. After application of bendiocarb at a dose of 500 µg/egg at 3 ED, we observed that in the viewing field of size 887.5 µm³ were 850 liver cells (with the mean number equal to one cell/µm³), any liver cells showed caspase activity of treatment embryos in comparison with the control.

In chicken embryos that were exposed to bendiocarb at 10 ED at doses of 800 µg/egg, was detected low caspases activity in comparison with the control. After application of bendiocarb at a dose of 800 µg/egg at 10ED, were found three (0.40%) liver cells with caspase activity contrary to the control with two (0.20%) caspase activity of the red stained cells (Fig. 6).

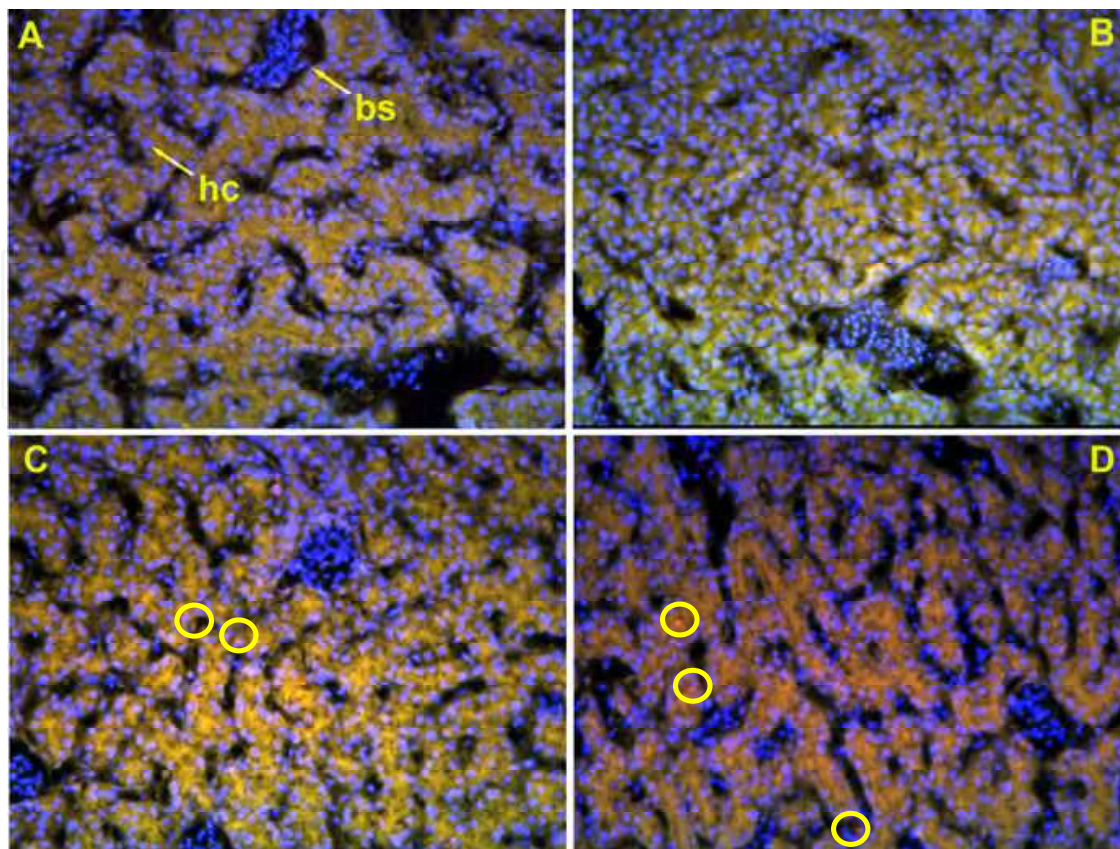


Fig. 6. Caspase activity of liver cells (in yellow circle) after application of BC on 3 ED (9 ED - A: control embryo, B: treatment embryo; 500 $\mu\text{g}/\text{egg}$) and 10 ED (17 ED - C: control embryo, D: treatment embryo; 800 $\mu\text{g}/\text{egg}$). blood sinusoid (bs); hepatocyte (hc) [stained immunohistochemically, 40x]

3.3.2 Central nervous system

Chicken embryos were administered bendiocarb at 3 ED at doses of 500 $\mu\text{g}/\text{egg}$. Among them, we observed 450 nerve cells (with the mean number of one nerve cell/ $2\mu\text{m}^3$, in the viewing field of size $887.5\mu\text{m}^3$. 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 $\mu\text{g}/\text{egg}$, 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. 7).

The chicken embryos exposed to bendiocarb showed low caspase activity of liver cells. The presence of apoptotic cells in the liver after application of bendiocarb may be related to physiological apoptosis occurring during embryogenesis. 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 cleavage 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 cells with caspase activity in comparison with the control. This applies to both the liver and CNS of chicken embryos.

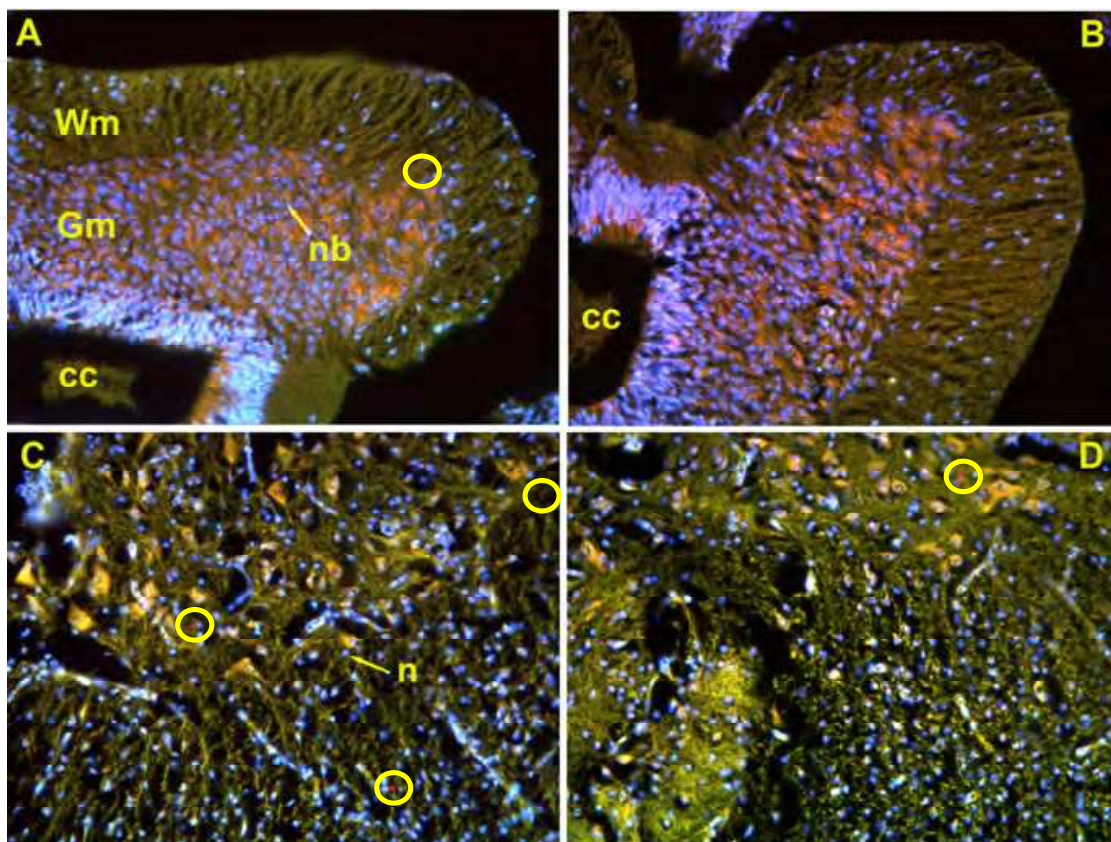


Fig. 7. Caspase activity of nervous cells (in white circle) after application of BC on 3 ED (9 ED - A: control, B: treatment embryo; 500 $\mu\text{g}/\text{egg}$) and 10 ED (17 ED - C: control, D: treatment embryo; 800 $\mu\text{g}/\text{egg}$). white matter (Wm); gray matter (Gm); central canal (cc); neuroblast (nb); nervous cell (n) [stained immunohistochemically, 40x]

Cell death with its well-known role in morphogenesis is an important characteristic of developing legs in chicken embryos (Dawd & 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 & 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 likely participate in formation of toes in amniotic embryos (chicken embryo, murine embryo and others) and also in humans (Dawd & 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 & Lockshin, 2002).

4. Conclusion

Pesticides represent the significant environmental contaminants, but often we cannot avoid their use in agriculture for plant protection and health of people and animals from diseases spread by vectors. Their extensive use raises the question about the toxicity of pesticides to non-target organisms, persistence, accumulation and combined effect with other agrochemicals. A great attention is paid to the study of impact of extraneous substances from external environment on humans and animal lately. It is mainly due to the high contamination of environment by chemical substances used in industry and agriculture. The most pesticides are not highly selective, and are generally toxic to many nontarget species, including humans. 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 liver or lung functions. Some are carcinogenic, other may cause reproductive toxicity or have endocrine disrupting properties. Many pesticides target the nervous system of insect pests. Because of the similarity of neurochemicals processes, these compounds are also likely to be neurotoxic to human. Prenatal development of the central cholinergic nervous system coupled with the important developmental roles played by ACh in both neural and non-neural tissues should make the vertebrate embryo especially vulnerable to the gestational effects of environmental neurotoxins that target cholinergic receptors or choline esterase. 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 extremely high toxicity (aldicarb - 0,8 mg/kg;). Given that the purity of environment and the negative impact of contamination on human and animal health is currently highly topical problem, we consider it necessary to pay attention to the action of pesticides on living organisms and thus to expand and acquire new knowledge about their potentially harmful effects. We thus conclude that bendiocarbamate does not possess a significant toxic 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.

5. Acknowledgement

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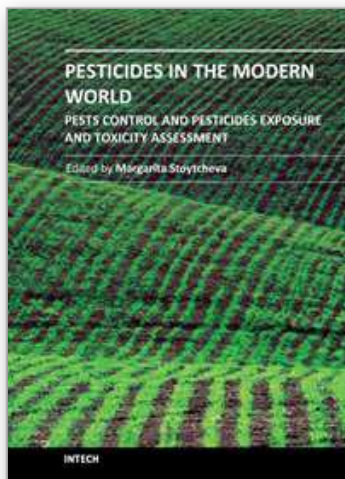
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The present book is a collection of selected original research articles and reviews providing adequate and up-to-date information related to pesticides control, assessment, and toxicity. The first section covers a large spectrum of issues associated with the ecological, molecular, and biotechnological approaches to the understanding of the biological control, the mechanism of the biocontrol agents action, and the related effects. Second section provides recent information on biomarkers currently used to evaluate pesticide exposure, effects, and genetic susceptibility of a number of organisms. Some antioxidant enzymes and vitamins as biochemical markers for pesticide toxicity are examined. The inhibition of the cholinesterases as a specific biomarker for organophosphate and carbamate pesticides is commented, too. The third book section addresses to a variety of pesticides toxic effects and related issues including: the molecular mechanisms involved in pesticides-induced toxicity, fish histopathological, physiological, and DNA changes provoked by pesticides exposure, anticoagulant rodenticides mode of action, the potential of the cholinesterase inhibiting organophosphorus and carbamate pesticides, the effects of pesticides on bumblebee, spiders and scorpions, the metabolic fate of the pesticide-derived aromatic amines, etc.

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