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Bendiocarb effect on liver and central nervous system in the chick embryo

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The aim of the study was to investigate toxicity of bendiocarb (2, 3-isopropyledene-dioxyphenyl methylcarbamate) to organs of chicken embryo. The toxic action of bendiocarb was observed on liver and central nervous system (CNS). Bendiocarb was administered to chicken embryos at embryonic day (ED) 3 in a dose 500 μg/egg and 10 ED (800 μg/egg). The observations showed no macroscopic or microscopic changes in the liver and CNS with either dose or day of incubation when the bendiocarb was administered. The liver and CNS were also investigated for caspase activity in relation to application of bendiocarb and no differences in the number of cells with caspase immunopositivity were observed in comparison with the control. The results obtained indicate that bendiocarb administered in the respective doses showed no toxicity to investigated organs. Furthermore, both at the early (3 ED) and the later (10 ED) stages of development no increase in numbers of apoptotic cells in chicken embryos was observed.

Keywords: Bendiocarb; chick embryo; toxicity.

Introduction

The carbamate compounds are a class of cholinesterase-inhibiting pesticides[1] and bendiocarb (2,3-isopropyledene-dioxyphenyl methylcarbamate) is the most widely used carbamate insecticide used to control disease vectors (mosquitoes, flies, 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.[2] Like other carbamate insecticides, bendiocarb is reversible inhibitor of acetylcholinesterase (AChE). The blockage of AChE caused by bendiocarb persists for approximately 24 hours and, subsequently, the situation returns to normal because the insecticide does not accumulate in mammalian tissues.[3] However, in relation to exposure to bendiocarb it was observed that the substance can easily pass from mother to the developing embryo.[4] Inhibition of AChE is linked with the pesticide mechanism of toxic action, irreversible or reversible bonding to the esteratic site of the enzyme and potentiation of cholinergic action on the nervous system.[5]

The bendiocarb can affect some biochemical and immunological parameters.[6] Acute intoxication with bendiocarb is manifested by peripheral nicotinic effect (muscle contraction, tachycardia, mydriasis etc.), peripheral muscarine effect (stimulation of glands, hypersalivation, nausea, vomitus etc.) and central effect (tremor, ataxy, convulsions and coma).[7]

Chronic intoxication with bendiocarb was investigated in a 2-year study on adult rats that were 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.[8] Adult rabbits that were administered bendiocarb per os for 90 days at the dose of 5 mg/kg/day, showed slight toxic effect of bendiocarb. However, no negative effect of bendiocarb was observed on formation of thymus structures.[2]

The nervous system of the chick embryo is formed from the 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 the

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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.[9] 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.[9]

Currently the oral LD_{50} of bendiocarb for hen is 137 mg/kg b.w.[10] 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 substances. Because of that the aim of the present study was to observe the effect of bendiocarb on chicken embryos. Chicken embryos are more sensitive than embryonic mammals to toxic substances because their metabolism is not influenced by maternal metabolism.

Materials and methods

Fertilized white Leghorn chicken eggs were purchased from the animal facility of the Institute of Molecular Genetics (Kolec, Czech Republic) and delivered via courier in a temperature controlled manner in order to ensure egg viability and quality (80 eggs). The eggs were incubated without storage blunt end up in a forced-draft constant-humidity incubator at 37.5°C with continuous rocking and relative humidity of 60%. Cytotoxicity to organs was observed by administration of bendiocarb at 3 ED and 10 ED. According to Hamburger and Hamilton,[11] the 3 ED represents the 19th stage of development while 10 ED represents the 36th stage of development. The eggs were opened by the modified "window technique".[12] The blunt end of eggs was cleaned with 70% alcohol and covered by a transparent adhesive tape. Subsequently, using serrated scissors (FST 14071-12), a window was cut for application of the respective solutions. The bendiocarb (96% purity, Bayer) was dissolved in acetone and diluted with sterile water intended for tissue cultures to obtain the required concentrations. The application dose per one egg was 200 μL, with acetone concentration equal to 10 μL/200 μL of the application dose. The doses of bendiocarb applied to chicken embryos at 3 ED were 500 μg/200 μL/egg. The doses of bendiocarb applied to chicken embryos at 10 ED were 800 μg/200 μL/egg. Identical volumes of acetone solution were applied to control embryos: sterile water for tissue cultures (1:10), with acetone concentration 10 μL/200 μL application dose. After cutting out the window in eggshell, the application dose was applied directly over the embryo on the top of inner shell membrane (membrana papyracea). After application of solutions the windows were covered with an electrical insulation adhesive tape (Tartan 1710) as described,[13], and the eggs were returned to a still draft incubator with the same temperature and humidity settings for re-incubation until the time of sampling.[14] The chicken embryos were observed during incubation and dead individuals were eliminated from the experiment.

The chicken embryos which were exposed to bendiocarb solution at 3 ED were dissected out of the membranes and weighted at 9 ED. Those which were exposed to bendiocarb solution at 10 ED were dissected out of the membranes and weighted at 17 ED. Subsequently, 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 3 ED, left 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 microscopical changes in the liver and CNS, part of the sections was stained with haematoxylin-eosin and the remaining sections were stained immunohistochemically for observation of caspase activity. 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. The caspase activity in the liver and CNS was observed 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 fluorescence microscope when using a suppression filter (465 nm) while the 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.

Results and discussion

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).

Liver

Comparison with the control showed neither macroscopic or 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. 1).

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. 2).

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.[8]

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.[2]

Male rats showed a significant increase in incidence of nuclear cataract related to bendiocarb dose (20 and 200 mg/kg).[15]

Caspase activity

Liver

The chicken embryos that were exposed to bendiocarb at 3 ED at concentrations of 500 μg/egg, showed low caspase 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 μm^3 were 850 liver cells (with the mean number equal to one cell/μm^3), any liver cells showed caspase activity of treatment embryos in comparison with the control.

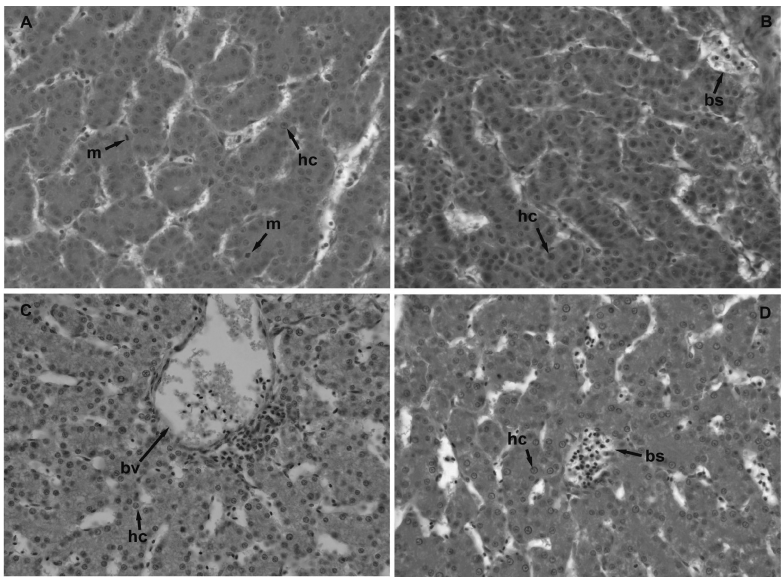

Fig. 1. Toxic action of bendiocarb (BC) on liver exposed on 3 embryonic day (ED) (9 ED—a: control embryo, b: treatment embryo; 500 μg/egg) and 10 ED (17 ED—c: control embryo, d: treatment embryo; 800 μg/egg). mitosis (m); hepatocyte (hc); blood vessel (bv); blood sinusoid (bs) [H-E, 60×].

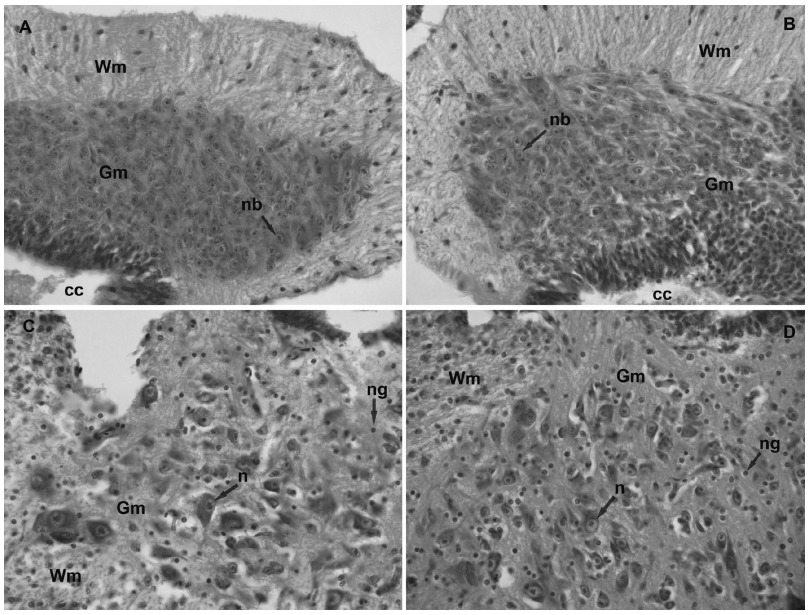

Fig. 2. Toxic action of bendiocarb (BC) on central nervous system (CNS) of chicken embryos exposed on 3 embryonic day (ED) (9 ED—a: control embryo, b: treatment embryo; 500 μg/egg) and 10 ED (17 ED—c: control embryo, d: treatment embryo; 800 μg/egg). White matter (Wm); gray matter (Gm); central canal (cc); neuroblast (nb); neuron (n); neuroglia (ng) [H-E, 60×].

In chicken embryos that were exposed to bendiocarb at 10 ED at doses of 800 μg/egg, was detected low caspase activity in comparison with the control. After application of bendiocarb at a dose of 800 μg/egg at 10 ED, 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. 3).

Central nervous system

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/2μm^3, in the viewing field of size 887.5μm^3. One cell (0.20%) showed caspase activity in comparison with the control.

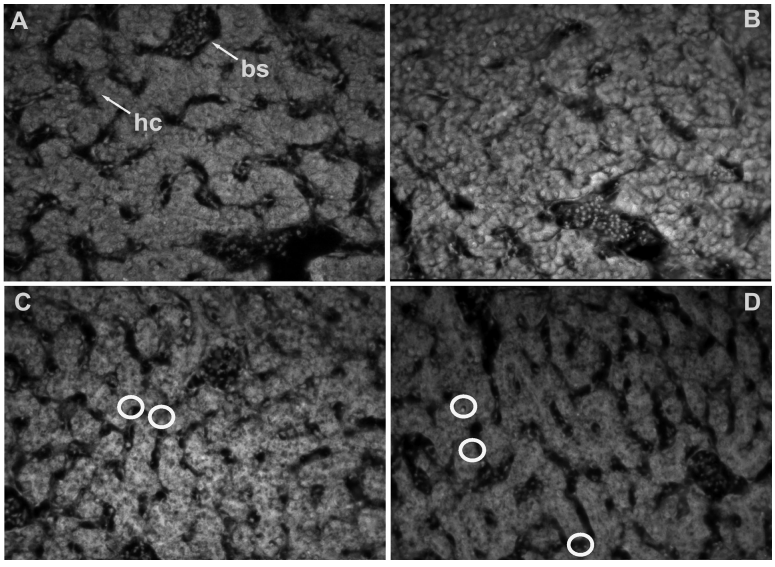

Fig. 3. Caspase activity of liver cells (in yellow circle) after application of bendiocarb (BC) on 3 embryonic days (ED) (9 ED—a: control embryo, b: treatment embryo; 500 μg/egg) and 10 ED (17 ED—c: control embryo, d: treatment embryo; 800 μg/egg). Blood sinusoid (bs); hepatocyte (hc) [stained immunohistochemically, 40×].

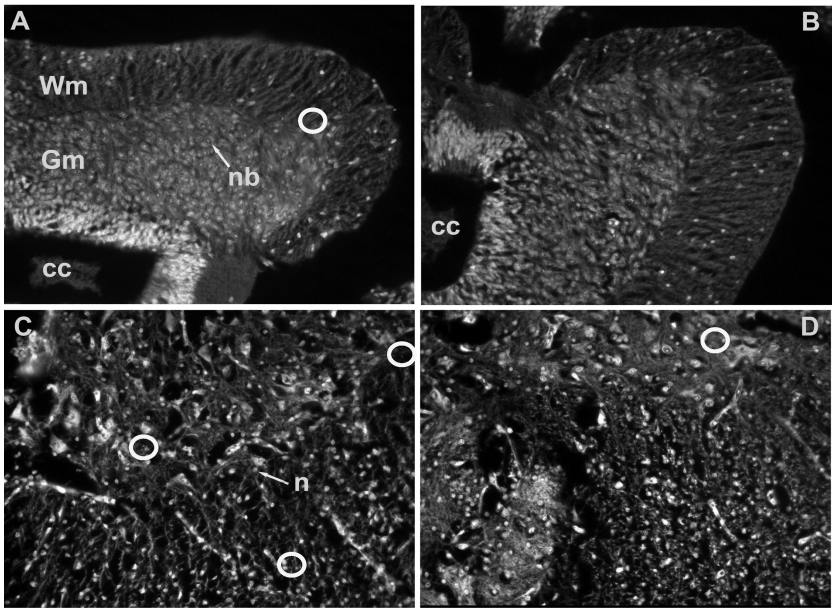

Fig. 4. Caspase activity of nervous cells (in white circle) after application of bendiocarb (BC) on 3 embryonic days (ED) (9 ED—a: control, b: treatment embryo; 500 μg/egg) and 10 ED (17 ED—c: control, d: treatment embryo; 800 μg/egg). White matter (Wm); gray matter (Gm); central canal (cc); neuroblast (nb); nervous cell (n) [stained immunohistochemically, 40×].

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. 4).

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.[16]

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.[17] 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.

Cell death with its well-known role in morphogenesis is an important characteristic of developing legs in chicken embryos.[18] 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.[19]

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 an in this way they likely participate in formation of toes in amniotic embryos (chicken embryo, murine embryo and others) and also in humans.[18] 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.[19]

Conclusion

We thus conclude that bendiocarb 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.

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