# Original Article

# Bendiocarbamate Toxicity in the Chick Embryo

(carbamate / pesticides / embryotoxicity / reproduction / development)

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**Abstract. Carbamate pesticides generally possess low toxicity for warm-blooded vertebrates, but developmental data are scarce. We have therefore evaluated embryotoxicity of choline esterase inhibitor bendiocarbamate in the chick embryo. The pesticide was dissolved in 5% acetone in distilled water and a volume of 200 µl was administered over the embryo through *membrana papyracea* on embryonic days 2, 3, 4, 5, and 10. Sampling was performed on embryonic day 10, while the embryos treated on embryonic day 10 were sampled on embryonic day 17. The toxicity of bendiocarbamate was fairly low, and $LD_{50}$ decreased with advancing development from 1 mg/embryo on embryonic day 2 to 29 mg on embryonic day 5. Malformations in surviving embryos were observed rarely (< 3 %) and occurred in both control and experimental groups. There was a mild but statistically significant dose-dependent reduction in body weight, most pronounced in the treatment on embryonic days 5 and 10, but the maximum difference from controls was below 15 %. A small but not significant increase in the number of positive cells was observed in the eye, limb buds, and the central nervous system of embryos treated on embryonic days 3 and 4 and examined after supravital whole-mount staining with Lysotracker Red for apoptosis. In agreement with previously published studies in other vertebrate animals, we conclude that bendiocarbamate does not possess significant toxicity in the avian embryo.**

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Abbreviations: CHEST – chick embryotoxicity screening test, ED – embryonic day(s).

## Introduction

Bendiocarbamate is a pesticide acting upon invertebrates by irreversibly blocking the activity of the enzyme cholinesterase, which is critical in allowing muscle relaxation by removing the neuromuscular mediator acetylcholine (Kristoff et al., 2006). It has relatively low toxicity in mammals and vertebrates in general, since it does not accumulate in their tissues (Siroťáková et al., 2005) and the cholinesterase activity returns to norm within 24 hours after acute exposure. Its acute oral toxicity ($LD_{50}$) was investigated in different adult mammals: rat 34–156 mg/kg, guinea pig 35 mg/kg, rabbit 35–40 mg/kg (WHO, 2005), and also in non-mammalian species such as birds: mallard duck 3.1 mg/kg, bobwhite quail 16 mg/kg, hen 137 mg/kg (WHO, 2007); fish 0.7–1.8 mg/l ($LC_{50}$, Hayes and Lawes, 1990); bee 0.1 µg/bee (Wright et al., 1981).

Chick embryo is a popular model for developmental pharmacological and toxicological studies. It is readily available, cost-efficient, and presents an alternative approach to the treatment of pregnant mammals. The concordance of data from the chick embryotoxicity screening test (CHEST) (Jelinek, 1977) and mammals is excellent, and it avoids the potentially confounding effect of different maternal metabolism between species by allowing 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.

In order to assess to the fullest extent the possible embryotoxic potential, we performed a detailed study of bendiocarbamate effects in the chick embryo. We found that the toxicity is fairly low (in the order of tens of grams per kg of embryonic weight, or tens of milligrams per kg of egg weight, respectively) with the earliest developmental stages being the most sensitive. In agreement with previously published data (Bendiocarb. Available at: http://www.pan-uk.org/pestnews/Actives/Bendiocarb.htm) we thus conclude that environmentally relevant concentrations (several orders of magnitude

lower, Bendiocarb Technical Fact Sheet – National Pesticide Information Center. Available at: http://npic.orst.edu/factsheets/bendiotech.pdf) do not seem to pose a significant risk to human reproduction.

## Material and Methods

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. The eggs were incubated without storage blunt end up in a forced-draft constant-humidity incubator at 37.5 °C with continuous rocking until embryonic days (ED) 2–10 of the (21-day) incubation period. Embryos that were growth-retarded or dysmorphic (twinning, exencephaly) at the time of treatment were excluded from further study.

On incubation days 2, 3, 4, 5, and 10, the eggs were windowed on the blunt end, and 200 µl of tested solutions with varied concentration of bendiocarbamate were applied directly over the embryo on the top of the inner shell membrane (*membrana papyracea*). Controls received the same volume of solvent alone (10 µl of acetone in 200 µl of water for injection). The ranges of concentrations and the total numbers of embryos are listed in Table 1. The windows were closed using electrical insulation tape as described (Sedmera et al., 2002), 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 (day 19 for applications on days 2–5, day 17 for incubation on day 10).

At the time of sampling, the 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. In a separate group of 11 embryos treated with 400 µg of bendiocarbamate on day 3 (estimated $LD_{50}$ for that stage, at which there is usually the highest incidence of malformations in survivors) and five controls, the sampling was performed at 24 and 48 h intervals for the purpose of whole-mount detection of apoptosis using Lysotracker Red (Invitrogen, Eugene, OR) (Schaefer et al., 2004). After staining, the embryos were fixed with 4% paraformaldehyde in PBS for 24 h at 4 °C, rinsed in PBS, dehydrated through graded ethanol series and cleared in benzyl alcohol-benzyl benzoate for examination in a

*Table.1 Bendiocarbamate embryotoxicity at different stages of development*

| ED | Dose (µg) | N | Dead | Mortality (%) | Malformed | Mean weight (g)* | Weight SD | *t*-test (P value) |
|---|---|---|---|---|---|---|---|---|
| 2 | 0 | 35 | 5 | 14 | 3 | 1.355 | 0.272 | |
| | 8 | 20 | 0 | 0 | 0 | 1.546 | 0.218 | 0.011 |
| | 80 | 22 | 1 | 5 | 1 | 1.399 | 0.378 | 0.627 |
| | 200 | 13 | 5 | 38 | 0 | 1.363 | 0.119 | 0.938 |
| | 400 | 11 | 5 | 46 | 0 | 1.305 | 0.081 | 0.660 |
| | 800 | 21 | 7 | 33 | 0 | 1.196 | 0.245 | 0.259 |
| | 1600 | 22 | 16 | 73 | 0 | 1.304 | 0.267 | 0.675 |
| 3 | 0 | 36 | 4 | 11 | 0 | 1.446 | 0.165 | |
| | 16 | 24 | 6 | 25 | 1 | 1.421 | 0.183 | 0.414 |
| | 160 | 19 | 2 | 11 | 0 | 1.536 | 0.111 | 0.123 |
| | 500 | 15 | 10 | 67 | 0 | 1.312 | 0.131 | 0.071 |
| | 1000 | 22 | 18 | 82 | 0 | 1.331 | 0.139 | 0.152 |
| | 1600 | 50 | 47 | 94 | 0 | 1.544 | 0.031 | 0.366 |
| 4 | 0 | 23 | 2 | 9 | 1 | 1.139 | 0.201 | |
| | 16 | 17 | 1 | 6 | 0 | 1.162 | 0.151 | 0.695 |
| | 160 | 18 | 1 | 6 | 0 | 1.196 | 0.119 | 0.304 |
| | 500 | 19 | 0 | 0 | 0 | 1.225 | 0.134 | 0.123 |
| | 1000 | 20 | 10 | 50 | 0 | 1.115 | 0.113 | 0.729 |
| | 1300 | 21 | 6 | 29 | 1 | 1.188 | 0.163 | 0.467 |
| | 1600 | 12 | 6 | 50 | 0 | 1.145 | 0.129 | 0.943 |
| 5 | 0 | 40 | 4 | 10 | 0 | 1.496 | 0.126 | |
| | 80 | 22 | 9 | 41 | 0 | 1.445 | 0.187 | 0.300 |
| | 160 | 21 | 11 | 52 | 1 | 1.635 | 0.220 | 0.120 |
| | 320 | 21 | 10 | 48 | 1 | 1.364 | 0.152 | 0.006 |
| | 500 | 23 | 1 | 4 | 0 | 1.306 | 0.133 | 0.000001 |
| | 1000 | 19 | 1 | 5 | 0 | 1.394 | 0.182 | 0.023 |
| | 1600 | 21 | 1 | 5 | 0 | 1.382 | 0.151 | 0.004 |
| 10 | 0 | 12 | 0 | 0 | 0 | 17.25 | 1.324 | |
| | 800 | 17 | 3 | 18 | 3 | 14.86 | 2.543 | 0.006 |
| | 1600 | 15 | 5 | 33 | 0 | 15.30 | 1.337 | 0.0009 |
| **Total** | | **651** | **197** | | **12** | | | |

*Wet weight of embryos sampled on ED10, except for application on ED10 when sampling was done on ED17.
P values considered statistically significant are in bold. The weight of embryos on incubation days 2, 3, 4, 5, and 10 according to Clark et al. (1986) are 5, 18, 80, 149, and 2820 mg, respectively.

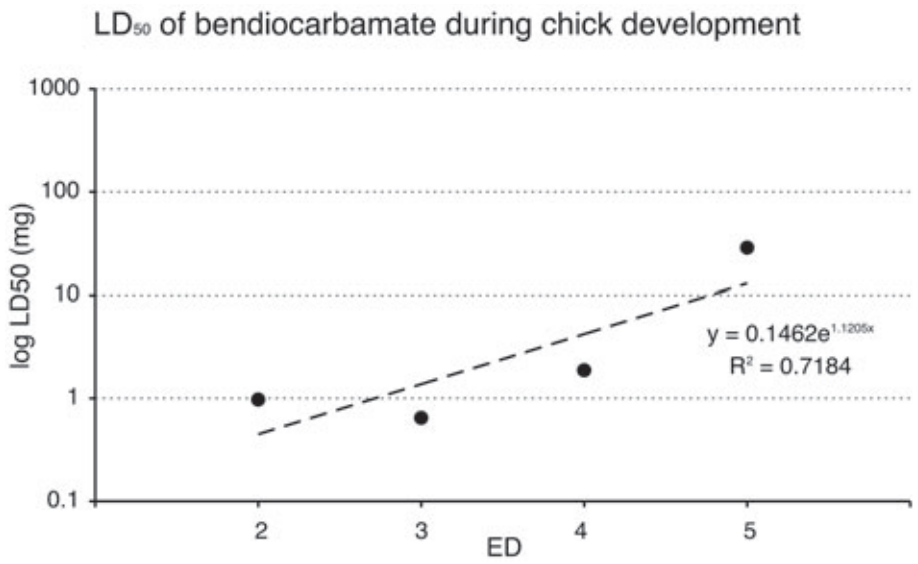


*Fig. 1.* $LD_{50}$ of bendiocarbamate increases with development. The graph is based on values from Table 1.

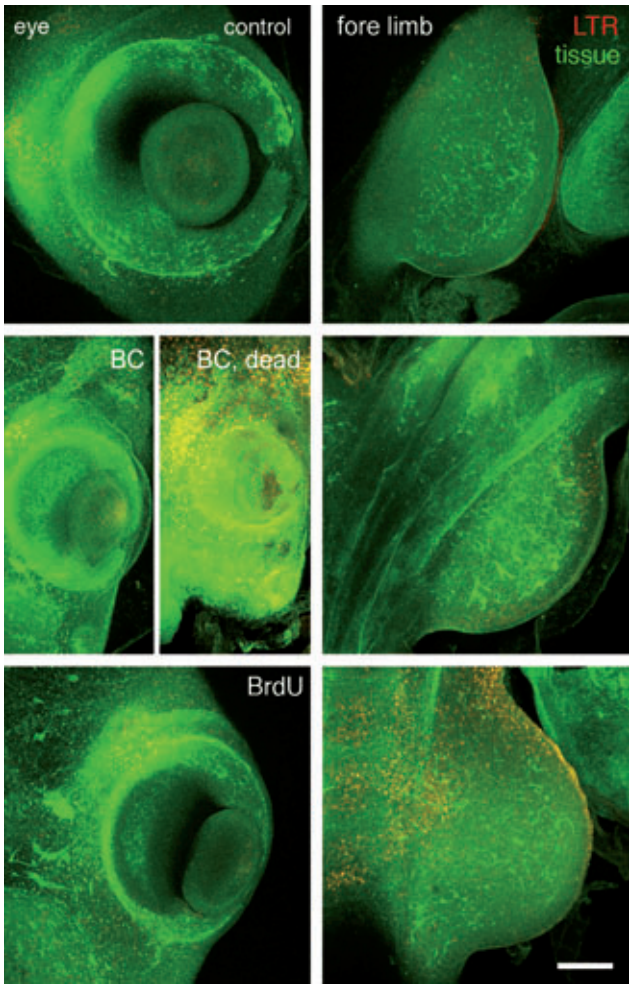


*Fig. 2*. Lysotracker Red (LTR) staining of ED4 embryos in control eye (a) and wing bud (b) and 24 h after administration of bendiocarbamate (BC) (c, d, e). A mild increase in the number of apoptotic cells is observed in the treated group, but a much more pronounced effect is visible in those treated with a teratogenic dose of bromoxeoxyuridine (BrDU) (positive control; f, g). Scale bar 100 µm.

confocal microscope (Miller et al., 2005). Images acquired in green and red channels of a Leica SPE confocal microscope (Leica Mikrosystems, Wetzlar, Germany) were processed using Adobe Photoshop.

Statistical comparison of differences among groups was performed using GraphPad Prism. Values of $P < 0.05$ were considered significant.

## Results

Total embryotoxicity of a single dose of bendiocarbamate was investigated on ED 2, 3, 4, 5, and 10. The embryolethality (expressed as $LD_{50}$) decreased with increasing age (Fig. 1), and could not be determined on day 10 because of solubility limits (200 g/l of acetone, but lower in mixture with water; too much of concentrated acetone is toxic to the embryo).

Table 1 lists the wet weight of embryos in different treatment groups. In general, administration of bendiocarbamate resulted in a small decrease in embryonic weight, with a clear correlation with the dose at later developmental stages (ED 5 and 10).

The malformations were observed sporadically in both treated and control group, with overall frequency below 3 %. Examples of malformations included defects of body wall, microphthalmia, anophthalmia, cleft beak, and general growth retardation. No specific pattern of malformations was observed among the treated embryos, irrespective of the dose.

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 apoptotic cells in the whole embryo 24 and 48 hours after application (Fig. 2). Since there was no specific pattern of malformations following bendiocarbamate 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 congeni-

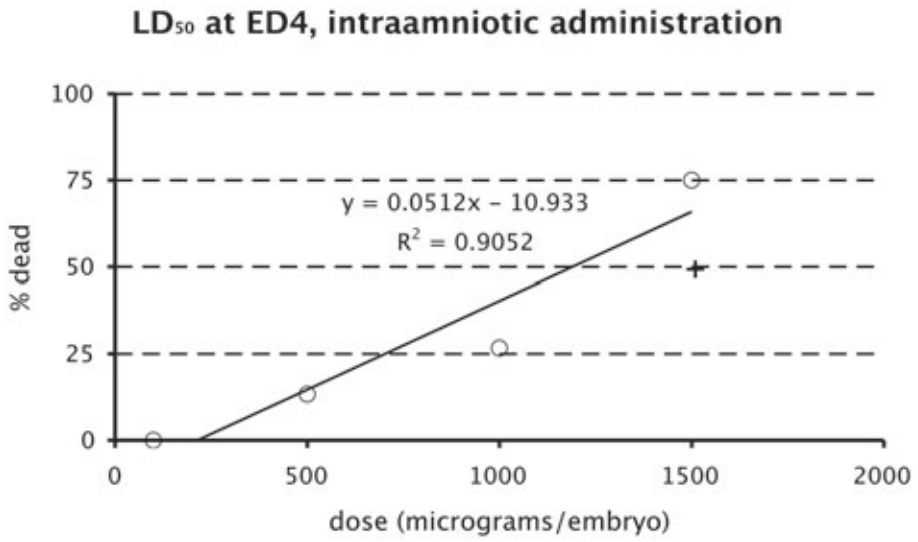


*Fig. 3*. Estimation of $LD_{50}$ on ED4 via intraamniotic administration. The resulting value of 1190 µg per embryo is very similar to that used with administration through the air sac membrane directly over the embryo (compare with Fig. 1).

tal anomalies (Sedmera and Novotna, 1994, and references therein). 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 bendiocarbamate-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.

## Discussion

This study provides the first detailed analysis of bendiocarbamate toxicity in the chick embryo for which (or any other embryo) there are no data in the literature. Acute oral toxicity of bendiocarbamate was investigated in adult mammals such as rat, guinea pig, or rabbit (WHO, 2005), together with $LD_{50}$ in non-mammalian species (e.g. mallard duck 3.1 mg/kg, bobwhite quail 16 mg/kg, hen 137 mg/kg (WHO, 2007), or fish 0.7–1.8 mg/l ($LC_{50}$, Hayes and 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 1; calculated $LD_{50}$ doses based on the embryonic weight are in the range 20–200 g/kg; considering the whole ~30 g egg as a distribution space, the range would be 24–924 mg/kg according to the 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.

Our method of administration through the papyraceous membrane differs from the original method of CHEST, which calls for either subgerminal or intraamniotic injection. We have chosen this route to overcome the combined problems with solubility and toxicity of higher volumes of concentrated acetone. 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 the intraamniotic route (Sedmera and Thompson, unpublished observations), but there is less procedure-related mortality and it is technically simpler (easier windowing and closing, yet keeping the advantage of visual control and possibility to exclude grossly abnormal embryos). To validate our approach against the intraamniotic injection, we performed a dose-response experiment on ED4 (Fig. 3). As expected, volumes over 10 µl of concentrated acetone were toxic to the embryo; nevertheless, we obtained a clear dose-response relationship and the estimated $LD_{50}$ was almost identical to that obtained with transpapyraceous application (1.2 mg per embryo; compare Figs. 3 and 1).

There were no specific malformations associated with bendiocarbamate exposure in our set of experiments. The ones encountered were also seen in the controls, and the frequency did not exceed 3 %, which is considered background noise in the pre-hatching chicks (Novotna and Jelinek, 1990; Novotna et al., 1994). We thus conclude that bendiocarbamate 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 foetus in mammals.

Apoptosis 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; Novotna et al., 1994). We have noted a mild increase in the number of apoptotic cells revealed by whole-mount staining with the vital dye, but it did not result in any congenital anomalies and was substantially lower than the

increase of cell death associated with e.g. bromodeoxyuridine embryotoxicity (Sedmera and Novotna, 1994) that does result in limb defects. It is possible that this mild reduction in cell number could underlie the small dose-dependent decrease of embryonic weights observed at the time of autopsy. The validity of using Lysotracker Red for whole-mount detection of cell death instead of previously used Nile Blue or Neutral Red was verified using bromodeoxyuridine in a parallel experiment. Embryos treated with 5 μg of bromodeoxyuridine (Sedmera and Novotna, 1994) served as internal control and showed a clearly increased frequency of cell death at 24 but not 48 h interval (Fig. 2 and data not shown).

Toxicity to specific target organs such as liver (involved in compound detoxification) or central nervous system (supposed to be most sensitive to inhibition of cholinesterase activity due to mediator accumulation) could be another manifestation of deleterious effects of bendiocarbamate in the chick embryo. Histological examination of these structures (Petrovova et al., 2009) did not show any significant morphological or immunohistochemical anomalies.

In conclusion, our systematic analysis of bendiocarbamate embryotoxic potential in the chick supports the earlier observations in other animal models, testifying to its relative safety for the embryo or foetus.

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