Toxicity hazard of organophosphate insecticide malathion identified by *in vitro* methods

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Abstract

OBJECTIVES: Malathion is generally not classified as toxic. However, the toxicity seems to be species-dependent. Local and systemic toxicity data for birds are rare, but a decrease of wild bird densities in areas where malathion was applied was reported. Aim of the study was to extend knowledge on malathion toxicity on cellular and organ level and to evaluate embryotoxicity and genotoxicity for birds using the chick embryo model HET-CAM.

METHODS: Skin and eye irritation was determined using reconstructed skin and eye cornea tissues and the chorioallantoic membrane of chick embryo to simulate conjunctiva. Cytotoxicity in 3T3 Balb/c fibroblast culture was determined to estimate acute systemic toxicity. Chick embryo model was further employed to evaluate acute embryotoxicity for birds (mortality and genotoxicity). Data were analysed by means of general linear models.

RESULTS: Malathion is not a skin and eye irritant. Cytotoxicity *in vitro* test provided LD_{50} value of 616 mg/kg suggesting higher toxic potential than is generally published based on *in vivo* tests on laboratory rodents. Embryotoxicity studies revealed dose and age dependent mortality of chick embryos. Genotoxicity was identified by means of micronucleus test in erythroid cells isolated from chorioallantois vascular system of chick embryos.

CONCLUSIONS: Using *in vitro* alternative toxicological methods, a higher toxic potential of malathion was demonstrated than is generally declared. An increased health and environmental hazard may occur in areas with intensive agricultural production. The environmental consequences of delayed effects and embryotoxicity for bird populations in areas exposed to organophosphate insecticides, such as malathion, are obvious.

INTRODUCTION

Organophosphate compounds are extensively used as pesticides. Malathion is a widely used organophosphate insecticide because of its relatively low toxicity to mammals and high selectivity towards insects compared to other organophosphate insecticides.

Organophosphates act as acetyl cholinesterase inhibitors and are active in a number of organs, such as the peripheral and central nervous systems, muscles, liver, pancreas and brain. Distinct

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data suggest inhibition of the thyroid gland hormones, degeneration of ovary follicle cells and increase in the incidence of fetus resorption (Brouwer *et al.* 1999). Recently, it has been reported that the metabolism of malathion produces reactive oxygen species that lead to the onset of oxidative stress (John *et al.* 2001; Fortunato *et al.* 2006) and that oxidative stress and DNA damage are possibly linked to pesticides-induced adverse health effects (Muniz *et al.* 2008; Santi *et al.* 2011). Other studies point to the effect of neurotoxicants on glucose metabolism that might be associated with increased risk of diabetes development (Montgomery *et al.* 2008; Rezga *et al.* 2010). Delayed effects and embryotoxicity may cause loss of recorded bird populations in areas where organophosphate insecticides were applied (Aktar *et al.* 2009).

Organophosphates exert toxic effects to pests through phosphorylation of serine residues in the active center of acetylcholinesterase, which leads to accumulation of acetylcholine. The majority of organophosphate insecticides, however, cause only slight inhibition of acetylcholinesterase unless they are activated. The selectivity and low toxicity of malathion is due primarily to mammals having a high level of carboxyesterases, enzymes that can hydrolyze malathion and its metabolites to non-toxic intermediates which can be eliminated from cells. Toxicity seems to be species-dependent, particularly dependent on carboxylesterase activity that breaks down the toxic malaoxon generated by oxidative sulphuration from malathion in the liver by cytochrome P450. Insects lack or have a low level of these esterases. Consequently, they can be severely affected by malathion bioactivated to malaoxon (Blasiak *et al.* 1999). Impurities of commercial products that inhibit carboxylesterase activity have the ability to potentiate the toxicity of malathion and therefore it is important to control the impurity profile of malathion products.

The present study aims to extend knowledge on malathion toxicity on cellular and organ levels. Number of experiments were performed using progressive alternative *in vitro* methods that model local and systemic toxicity, including cytotoxicity, embryotoxicity and genotoxicity evaluation. The effects of chemically pure malathion were tested by means of *in vitro* methods, such as skin and eye *in vitro* irritation assays (EpiDerm^{TM} and EpiOcular^{TM} reconstructed tissue models) and 3T3 fibroblast cell culture for risk assessment of systemic toxicity, i.e. estimation of lethal dose for 50% of animals (LD_{50}). The hen´s egg test on chorioallantoic membrane (HET-CAM) was employed to assess chick embryo survival (mortality) in a broad range of malathion dosage with two modes of application (into the air cavity or amnion) in two groups of chick embryos of different age. Hen's egg test was used also for genotoxicity assessment evaluating micronuclei induction in erythroid cells isolated from chorioallantois vascular system of embryo (hen´s egg test for micronucleus induction, HET-MN). The suitability of chick embryo model to assess acute embryotoxicity for birds (mortality and genotoxicity) was further examined.

MATERIAL AND METHODS

Chemicals

Malathion was obtained from Sigma-Aldrich (CAS 121-75-5).

Skin and eye irritation

Irritation potential was determined using reconstructed skin and eye cornea tissues EpiDerm^{TM} and EpiOcular^{TM} produced by MatTek Corporation (Ashland, MA, USA), and HET-CAM assay.

Skin Irritation Assay – EpiDerm™ Tissue Model EPI-200.

The EpiDerm™ Assay was conducted according to protocol "*IN VITRO* EpiDerm™ SKIN IRRITATION TEST EPI-200-SIT" (MatTek Corporation 2009, http://www.mattek.com) using an organotypic model of the human epidermis cultured from normal human keratinocytes. Upon receipt, the EpiDerm™ tissues were stored at 2–8 °C. Before use the tissues were equilibrated overnight at 37°C, 5% CO_2 in assay medium. Malathion was applied neat to the surface of triplicate tissues in the amount of 30 µl for 60 min. Sterile deionized water served as negative control, 5% aq SDS (Sodium Dodecyl Sulphate, SIGMA-Aldrich, CAS 151-21-3) was used as positive control. After exposure the tissues were extensively rinsed in PBS (phosphate buffered saline) and transferred into 1 ml of fresh medium for 42 h post exposure incubation. Following post-incubation, the MTT assay for cell viability was performed. The tissues were transferred to 24-well plates containing MTT medium (Thiazolyl Blue, CAS 298-93-1, concentration 1 mg/ml of culture medium). After a 3 h incubation, the blue formazan salt formed by cellular mitochondria was extracted with 2 ml of isopropanol per well and the optical density was determined by spectrophotometer Varian Cary UV-VIS 1E (Varian Inc., USA) at 570 nm. The prediction model developed by MatTek Corporation states that if the mean relative tissue viability of three individual tissues exposed to the test substance is reduced below 50% of the mean viability of the negative controls, the chemical is classified as an irritant (R38); viability >50% indicates non-irritant (no label).

Ocular Irritation Assay – EpiOcular™ Tissue Model OCL-200.

The EpiOcular™ Assay was performed according to protocol "EpiOcular™ EIT FOR THE PREDICTION OF ACUTE OCULAR IRRITATION OF CHEMICALS" issued by the tissue model supplier (MatTek Corporation 2011, http://www.mattek.com). Upon receipt, the EpiOcular^{TM} tissues were stored at 2–8 °C. Before use the tissues were equilibrated overnight at 37 °C, 5% CO_2 in assay medium. Malathion was applied undi-

luted and diluted in saline (1% and 25%) to the surface of duplicate tissues, pretreated with 20 µl PBS for 30 min, in the amount of 50 µl for 30 min. Sterile deionized water served as negative control, methyl acetate (SIGMA-Aldrich, CAS 79-20-9) was used as positive control. After exposure the tissues were extensively rinsed in PBS and allowed to post-soak submerged in assay medium for 12 min. Next, the tissues were transferred into 1 ml of fresh medium for 2 h post exposure incubation. Following post-incubation, the MTT assay for cell viability was performed. The tissues were transferred to 24-well plates containing MTT medium (1 mg/ml). After a 3 h incubation, the blue formazan salt formed by cellular mitochondria was extracted with 2 ml of isopropanol per well and the optical density was determined by spectrophotometer Varian Cary UV-VIS 1E (Varian Inc., USA) at 570 nm. The prediction model developed by MatTek Corporation, based on the test article-treated tissues viability relative to negative control-treated tissue viability, introduces a single cut-off in relative survival for classification of eye irritation: viabilty ≤60% = irritant (I) (R36 and R41); viability >60% = non-classified (NC).

HET-CAM test

HET-CAM test according to the INVITTOX Protocol No.47 (http://ecvam-dbalm.jrc.ec.europa.eu) was performed to simulate effects on eye mucosa using the rich vascular system of chorioallantoic membrane of chicken embryo. Nine-day fertile eggs supplied by an accredited producer (Habry Hatcheries, eggs COBB 500, ROSS 308) were employed in the test. Viability of the embryos was checked on a regular basis. The integrity of chorioallantoic membrane was assessed after removing the eggshell around the air pocket. The eggs were then exposed to malathion applied on the chorioallantoic membrane either undiluted or in PBS/water emulsions. First appearance (during 5 min.) of haemorrhage (H), lysis (L)/vasoconstriction (V) and coagulation (C) in seconds was determined (by microscopic evaluation) after application. Irritation Score (IS) was calculated as:

$$IS = (301-H)\times 5/300+(301-(L/V))\times 7/300+(301-C)\times 9/300.$$

Classification on the basis of calculated IS: non-irritant 0.0–0.9, slight irritant 1.0–4.9, moderate irritant 5.0–8.9, severe irritant 9.0–21.0.

Balb/c 3T3 NRU cytotoxicity test – a screen for low toxicity (LD_{50} > 2000 mg/kg)

The test was performed according to the ICCVAM 2006 Report (ICCVAM 2006). Briefly, Balb/c 3T3 fibroblasts (clone L1, ECACC, Salisbury, UK) were subcultured for 24 h in Dulbecco´s Modified Medium supplemented with bovine serum (10%) until reaching confluence ≤50%. Eight selected concentrations of malathion, diluted in DMSO (final maximal solvent concentration 0.5% v/v in culture medium) were tested with six replicates per concentration. After 24 and 48 h incubation the Neutral Red Uptake (i.e. cell viability) was determined fluorimetrically (FLX800TBI reader, Biotek, USA). The viability of cell cultures treated with the experimental samples was expressed as percentage of the viability of the baseline cell culture control (treated with 0.05% DMSO in culture medium). Finally, the concentration which led to a 50% reduction in cell growth (IC_{50}) was estimated from the concentration-response curve.

The predicted LD_{50} for lethal oral toxicity in rats was calculated using the formula: log LD_{50} (mg/kg) = 0.372logIC_{50} (µg/ml) + 2.024.

Embryotoxicity

The model of chick embryo was employed for evaluation of acute embryotoxicity for birds (mortality and genotoxicity).

Mortality was evaluated morphologically in two groups of chick embryos aged 4 days (ED4) or 8 days (ED8), evaluated up to 3 days after application performed on Day 4, resp. Day 8. Six fertilized eggs were employed for each dose. Doses were ranging from 3 to 30,000 µg/egg, applied in water/saline emulsion into the air cavity or amnion.

Genotoxicity was evaluated using the method of Wolf *et al.* 2008. Air dried blood smear (blood taken from arteria umbilicalis) was stained by May-Grünwald solution and observed under bright field microscopy. Slides were examined using the microscope Olympus BH2-RFCA. Morphological evaluation included the number of erythrocytes with micronuclei and the number of mitosis per 1,000 erythroid cells.

Statistics

Data were analysed by means of regression analyses and analyses of variance. Variables were log-transformed when needed to attain normality and homoscedasticity. Statistical significance was assumed at $p<0.05$. The data were collected and processed using PASW Statistics 18 programme package.

RESULTS

Skin and eye irritation

Malathion was not found to be a skin and eye irritant. The viability of EpiDerm™ tissue model after application of undiluted malathion was detected as 89.2%. The viability of EpiOcular™ tissue model after exposure to malathion was higher than 60% in all concentrations tested, including undiluted malathion (viability 98.2% for 1% dilution, viability 92.3% for 25% dilution and viability 82.5% for malathion applied neat). No potential of irritation was demonstrated even on the chorioallantoic membrane of chick embryo simulating eye mucosa. Eye irritation score was lower than 0.5 for all the concentrations tested (1–100%), including application of undiluted malathion.

Acute toxicity in vitro

LD_{50} value calculated by formula: log LD_{50} (mg/kg) = 0.372logIC_{50} (μg/ml) + 2.024 using the cytotoxicity *in vitro* data was determined as 616 mg/kg for 48 h exposure, resp. 467 mg/kg for 24 h exposure. According to the *in vitro* acute toxicity data, malathion can't be classified as non-toxic, i.e. not corresponding to category of not classified chemicals exhibiting LD_{50} *in vivo* >2000 mg/kg.

Embryotoxicity

Mortality of chick embryos was dose dependent, higher susceptibility was demonstrated for 4 day embryos. Results are summarized in Figures 1–2. The statistical analysis based on general linear model (GLM) proved significant dependence of mortality on the dose of malathion and the age of embryos ($p<0.01$).

Compiled LD_{50} values with respect to animal species (obtained from literature) expressed in mg per kg, including the mortality LD_{50} data related to the place of malathion application and age of embryos obtained from our present study, are shown in Figure 3.

Genotoxicity studies revealed significant changes in numbers of micronuclei and mitosis in erythroid cells in a group of embryos aged 8 days at malathion application and evaluated at Day 11 of the embryo age. Both induction of micronuclei and reduction of mitosis were dose dependent when malathion was administred into the air cavity. The dose dependent increased numbers of micronuclei and the decreased number of mitosis reached statistical significance ($p<0.05$). Data are shown in Figures 4–5.

DISCUSSION

The acute toxicity data of agrochemicals are currently obtained by means of animal experiments that are conducted in accordance with the European Commission binding legislation (EC 2006, EC 2008). The specific request of the so called REACH Regulation is to use alternative *in vitro* methods instead of *in vivo* tests. This request is further supported by provisions of Directive 2010/63/EU on the protection of animals used for scientific purposes. Sufficient data on genotoxicity and developmental toxicity of commonly used substances are frequently missing, or they were not generated in accordance with the mandatory test methods. Moreover, due to the different pharmacokinetics of laboratory animals, the animal experimental data do not entirely correspond to those of man. Broader use of *in vitro* methods based on cells of human origin is thus highly desirable in order to confirm their predictive capacity and applicability domain. Our study contributed to this area and provided new toxicity data on malathion obtained by means of alternative *in vitro* methods.

Malathion is known for low toxic effect in man and mammals in general, but it is highly toxic for other vertebrates as fish and for distinct invertebrates. The acute

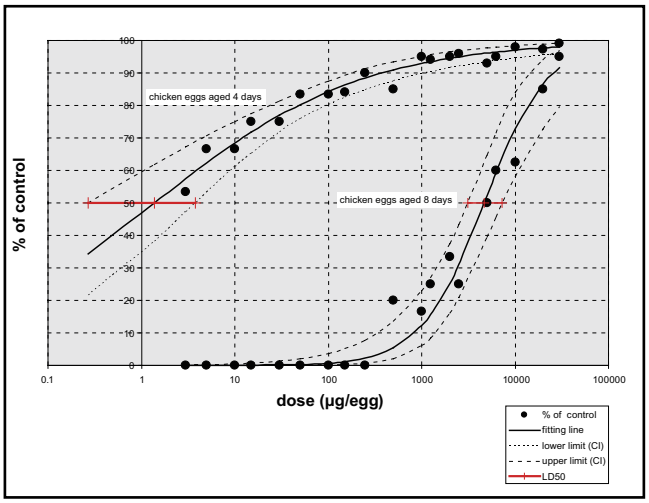

Fig. 1. Embryo mortality resulting from injection of malathion into amnion of incubated chicken eggs aged 4 days (LD_{50} = 1.38 μg/egg (95% CI: 0.27–3.80 μg/egg) and 8 days (LD_{50} = 4657 μg/egg (95% CI: 3141–7311 μg/egg).

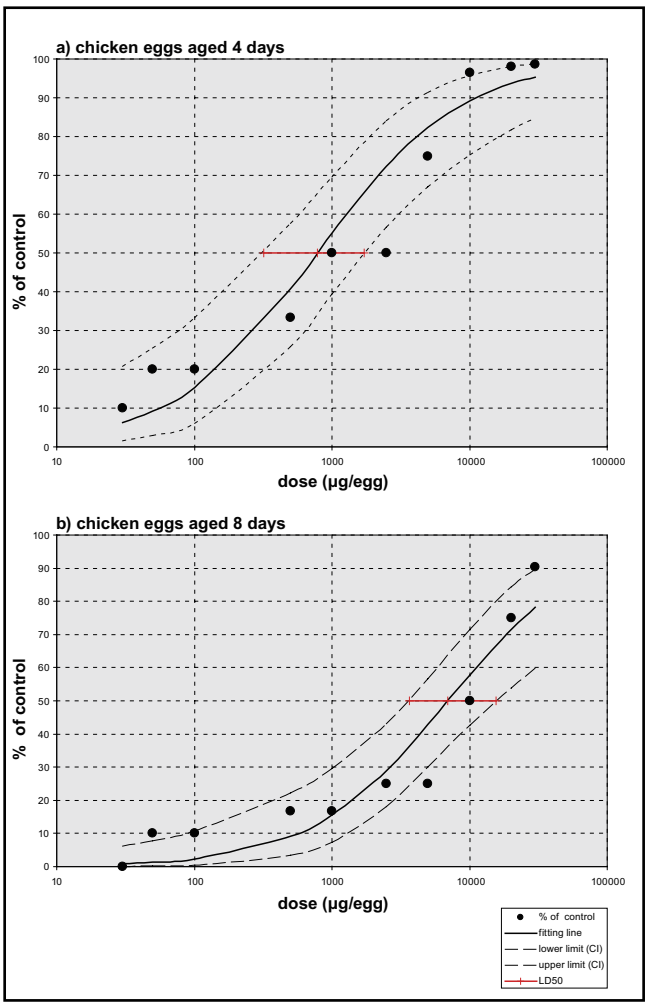

Fig.2. Embryo mortality resulting from malathion administration into air cavities of incubated chicken eggs aged: a) 4 days (LD_{50} = 783 μg/egg (95% CI: 318–1720 μg/egg), b) 8 days (LD_{50} = 6978 μg/egg (95% CI: 3636–15581 μg/egg).

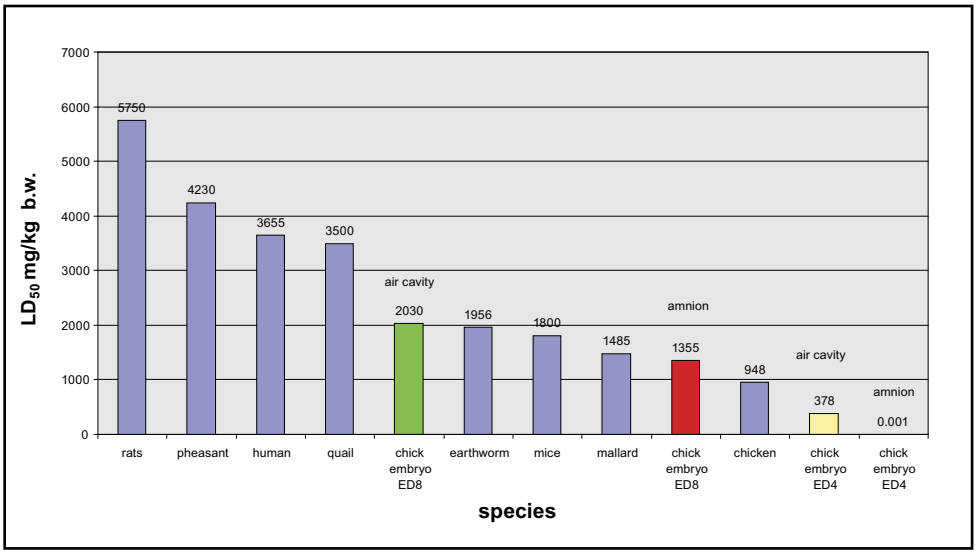

Fig. 3. LD_{50} values of malathion obtained from literature with respect to animal species and chick embryo mortality LD_{50} data obtained in the presented study, related to the place of application and chick embryo age (Day 4 and Day 8).

oral toxicity of malathion for rodents (rat) was set over 5500 mg/kg (Kamrin 1997). Reported data on acute systemic toxicity for other species summarized in Figure 3 in general exceed 2000 mg/kg (Ginter *et al.* 2002). Our data on mortality of chick embryos *in vitro* were significantly dependent not only on the age of embryos, but also on the place of application. The mortality was significantly higher for 4 days aged embryos than for 8 days aged embryos or when malathion was applied into the amnion compared to air cavity (Figures 1–2). For malathion applied in the air cavity the hen´s chick embryo test estimated LD_{50} values of 783 µg/egg for 8 day aged embryos and LD_{50} of 6978 µg/egg for 4 days aged embryos. When malathion was applied into the amnion, the LD_{50} was only 1.38 µg/egg for 4 days aged embryos, resp. 783 µg/egg for 8 days aged embryos. When LD_{50} was estimated using the cytototoxicity test *in vitro* the calculated LD_{50} values were 616 mg/kg for 48 h exposure, resp. 467 mg/kg for 24 h exposure, i.e. malathion should be classified as toxic. Although the screening cytotoxicity cell culture assay was extensively used for LD_{50} prediction (Clothier *et al.* 2008, OECD 2010), it has been previously noticed that the 3T3 NRU test method has certain limitations. In case of chemicals that exhibit toxicity only after being metabolised, their toxicity may be underpredicted as 3T3 Balb/c fibroblasts have only a limited metabolic capacity. For distinct classes of chemicals that exert their toxic effect by specific mechanisms not present in 3T3 cells, such as neurotoxic and cardiotoxic chemicals, the acute toxicity could be underpredicted using the cytotoxicity 3T3 NRU assay (OECD 2010). Malathion has been also reported to decrease the number of lysosomes and may thus affect NRU binding and retention in the cell (Bakr 2012). In case of a neurotoxic substance such as malathion, the *in vitro* LD_{50} value of 616 mg/kg suggests significantly higher acute toxicity than is usually reported for man, i.e. cca 3655 mg/kg (Talcott *et al.* 1979), especially considering vulnerable human population groups (children, seniors). The LD_{50} value of 616 mg/kg obtained *in vitro* corresponds to our results of chick embryotoxicity or to the reported acute toxicity for chicken (Ginter *et al.* 2002).

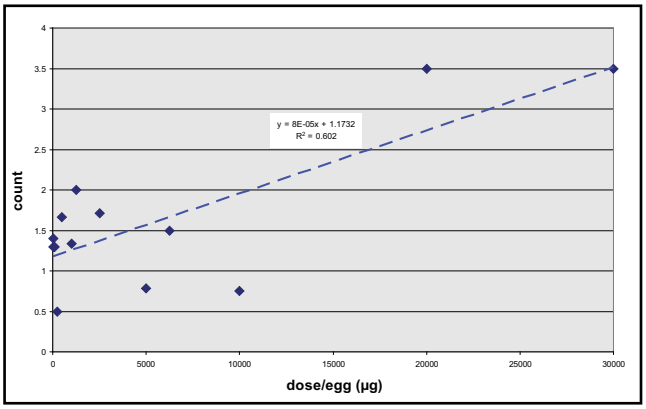

Fig. 4. Micronuclei – average number in erythroid cells dependent on malathion dose, 8 day embryos, application in the air cavity.

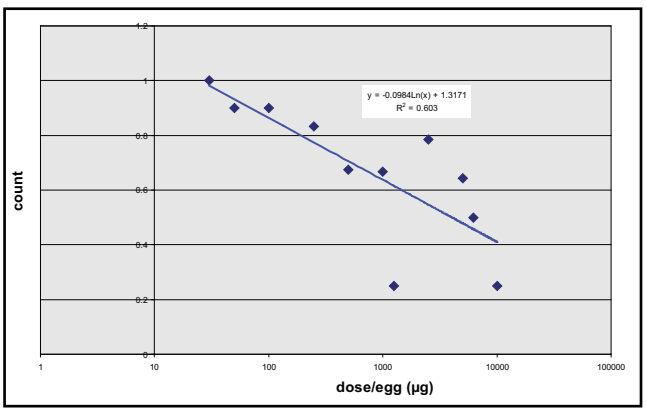

Fig. 5. Mitosis – average number in erythroid cells dependent on malathion dose, 8 day embryos, application in the air cavity.

No irritation potential of malathion was detected using the reconstructed models of the human skin and eye cornea tissues *in vitro* (EpiDerm™, EpiOcular™), in accordance with available data (IUCLID, http://esis.jrc.ec.europa.eu). No irritation was recorded even when we applied undiluted malathion on the chorioallantoic membrane of chick embryo simulating the eye mucosa in the HET-CAM test.

In our embryotoxicity studies we aimed to determine the LD_{50} of neurotoxic malathion using the *in vitro* model of chick embryo. We observed remarkable differences in the malathion embryotoxicity depending on the mode of its administration and the age of embryos. Our results are in agreement with mortality data (LD_{50}) of chick embryos exposed to malathion published by Pourmirza *et al.* 2000, who also reported toxic effects of malathion injected into the yolk sac of fertile chicken eggs. He determined the LD_{50} for chicken embryo at 3.59 mg/egg, which is the value in the same toxicity range as our finding when malathion was applied into the amnion of incubated chicken eggs aged 8 days (4.66 mg/egg).

Chick embryo has complex metabolic competence, it may serve as a good model system for cytotoxic/genotoxic testing, and it is capable of covering metabolic activation and deactivation of xenobiotics. We have investigated the genotoxic potential of malathion in the hen's egg test for micronucleus induction. The micronucleus test is one of the *in vivo* assays widely used to evaluate DNA damage caused by clastogenic and aneugenic substances and plays an important role in genetic toxicology in assessing the genotoxic potential of compounds. The formation of micronuclei in the erythrocytes of peripheral blood of incubated hen's eggs is described as a measure of genotoxicity, and demonstrated a positive response after administration of direct acting mutagens and promutagens (Wolf and Luepke 1997). Our present results document the increased number of micronuclei and decreased number of mitosis. The micronucleus assay basically requires a proof of proliferation since the formation of micronuclei /MN) strictly depends upon mitosis. An antiproliferative effect of the test substance is prone to produce a false negative result as a consequence of suppressed cell division (Wolf *et al.* 2002). In addition, under a prominent cytotoxic effect positive MN scoring may be highly difficult due to unusual cell morphology (Wolf and Luepke 1997). Under a lower cytotoxic effect (without the morphological appearance of nuclear aberrations which seems to be the case of malathion) the scoring of the preparations is relatively easy and enables the detection of genotoxicity potential. Futhermore, some genotoxic compounds are not detectable by *in vitro* genotoxicity tests unless the concentrations tested induce a certain degree of cytotoxicity. In this case genotoxic effects occur once a certain concentration threshold of cytotoxicity of a compound is reached. We assume that the mitogenic effect may be associated with mechanisms other than direct cytotoxicity and is not simply related to a lower survival of cells (cytotoxicity). Under the conditions of HET-MN test, our unexpected findings may thus be related to specific dose and time related systemic action of malathion in the living chick embryo. Another experimental study confirmed the ability of a mixture of pesticides (malathion, chlorpyriphos) to cause significant DNA damage demonstrated as chromosomal aberrations and sister chromatid exchanges persisting in a group of exposed workers even 8 months after the end of exposure (Blasiak *et al.* 1999, OMARI 2009, 2011). Moore *et al.* 2010 elucidated the role of oxidative stress in malathion-induced toxicity and genotoxicity, using human liver carcinoma (HepG2) cells. Our results on malathion potential of genotoxicity might thus be in accordance with the data on malathion genotoxicity/cytotoxicity mentioned above.

The crucial factor for any toxic effect is the substance bioavailability in the target tissue. Assessing the susceptibility of any organism to a toxicant, the amount of toxin per body unit is substantial (Robertson and Preisler 1992). In our experiments, the period from malathion application to the observed embryo death can be attributed to the transport of malathion accross tissues and its bioavalibility to embryo organs. The other factor may be higher susceptibility of the embryo to toxic effects in earlier stages of development. In addition, we have observed a relatively lower degree of chick embryo mortality at the highest doses of malathion when malathion was administrated into the air cavity. Interestingly, during our *in vitro* experiments using 3T3 fibroblast line by means of light microscopy we noticed "malathion floculation" in high concentrations above 1%. It could be attributed to physical properties and behavior of malathion in polar solutions under the conditions of our experiments. Water solubility of malathion was set at 148.2 mg/l at 25 °C, i.e. cca 0.015%. Malathion is an abiotic enviromental component and its stability in water was set at 49 hrs (t1/2) at 25 °C (IUCLID, http://esis.jrc.ec.europa.eu). The floculation phenomenon should be taken in consideration when assessing time dependent malathion toxicity/genotoxicity or whenever using aquatic or other polar emulsions with higher concentrations of malathion.

Compared to other cholinesterase inhibitors of the same group (e.g. paraoxon) malathion is considered as relatively safe for mammals, if used under foreseeable conditions. This fact can be attributed to the rapid hydrolytic degradation by carboxylesterases. Malathion is used as an effective insecticide in many developing countries as well as against most household pests or human body lice. However, some recent studies suggest that malathion may have the potential to induce mutagenic and/or carcinogenic effects under chronic exposure (Reus *et al.* 2008).

CONCLUSIONS

Using *in vitro* alternative toxicological methods, a higher toxic potential of malathion was demonstrated than is generally declared in literature, based on conventional *in vivo* tests on laboratory rodents. According to the *in vitro* acute toxicity data, malathion should be classified as toxic. In our experiments, the mortality rate and the embryotoxic effect of malathion detected in chick embryo model was found to be significantly dose dependent. Borderline genotoxicity was identified by means of micronucleus test in erythroid cells isolated from chorioallantois vascular system of chick embryos.

An increased health and environmental hazard may occur in areas with intensive agricultural production. The carboxylesterases activity differs across species and malathion toxicity is dependent on its foreseen hydrolytic degradation. The environmental consequences of delayed effects and embryotoxicity for bird populations in areas exposed to the organophosphate insecticides, such as malathion, are obvious.

Potential Conflicts of Interest: *None disclosed.*

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