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Comparative assessment of the nature of the combined action of insecticides: different methodical approaches

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ABSTRACT

Introduction. *Pesticide mixtures containing insecticide methomyl are commonly used in agriculture. However, the interaction of their active ingredients in a mixture has been insufficiently studied.*

The purpose of the study *was to evaluate the nature of the combined action of a mixture of insecticides methomyl (carbamate) and bifenthrin (pyrethroid) using various methods: a classical model (orthogonal planning of the experiment using nonlinear variables); a generalized linear regression model for the binomial distribution (probit regression); and an isobologram. The study was conducted at the Federal Scientific Center of Hygiene named after F.F. Erisman of the Federal Service for Supervision in Protection of the Rights of Consumer and Man Wellbeing, according to the protocol approved by the local bioethical commission.*

Materials and methods. *Ninety male outbred rats were administered 9 combined doses of the insecticides (combinations of LD_{16}, LD_{33} and LD_{50} for the individual compounds, as devised from a preliminary study).*

Results. *Impaired coordination, tremor of the limbs and head, turning into convulsions, rapid (shallow) breathing were observed in all groups. The data on mortality as used to construct 2 mathematical models, as well as to construct an isobologram.*

Limitations. *The study was limited to studying the indicators of acute oral toxicity, without taking into account the possible repeated exposure.*

Conclusion. *The models showed that the additive effect for the combination of methomyl and bifenthrin was only observed for combinations when the individual dose of bifenthrin was equal to or greater than 45 mg/kg b.w. (LD_{33}) and the dose of methomyl was less than 30 mg/kg b.w., as all animals receiving a dose of 30 mg/kg b.w. of methomyl (LD_{50}) in the mixture died. Therefore, the dose of methomyl dictated the toxic effect of the mixture, which should be accounted for in hygienic regulation of methomyl-containing formulations.*

Keywords: *combined action; pesticide; xenobiotic; interaction; mixture effect*

Compliance with ethical standards. *The study was approved by the Bioethical Commission of the of the Federal Scientific Center of Hygiene named after F.F. Erisman of the Federal Service for Supervision in Protection of the Rights of Consumer and Man Wellbeing, protocol № 01/22 dated 27.10.2022.*

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Сравнительная оценка характера комбинированного действия инсектицидов: разные методические подходы

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РЕЗЮМЕ

Введение. *Комбинированные препараты, содержащие метомил, широко используются в сельском хозяйстве, однако взаимодействие их активных ингредиентов в смесях изучено недостаточно.*

Цель исследования *– оценка комбинированного действия смеси инсектицидов метомила (карбамат) и бифентрина (пиретроид) с использованием различных методов: классической модели (ортогональное планирование эксперимента с нелинейными переменными), обобщённой линейной регрессионной модели для биномиального распределения (пробит-регрессия) и изоболограммы. Исследование проведено во ФБУН «ФНЦГ им. Ф.Ф. Эрисмана» Роспотребнадзора в соответствии с протоколом, утверждённым комиссией по биоэтике.*

Материалы и методы. *Беспородным крысам-самцам (n = 90) вводили девять комбинированных доз инсектицидов (комбинации LD_{16}, LD_{33} и LD_{50} для отдельных соединений, разработанные на основе предварительного исследования).*

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Оригинальная статья

Результаты. *Во всех группах у опытных животных после введения наблюдались нарушение координации, тремор конечностей и головы, переходящий в судороги, учащённое (поверхностное) дыхание. Данные о смертности в каждой из групп использовали для построения двух математических моделей и изоболограммы.*

Ограничения исследования: *изучение показателей острой пероральной токсичности без учёта возможной повторной экспозиции.*

Заключение. *Использованные модели показали, что аддитивный эффект комбинации метомила и бифентрина характерен только для сочетаний, в которых индивидуальная доза бифентрина была равна или превышала 45 мг/кг массы тела (LD_{33}), а доза метомила составляла менее 30 мг/кг массы тела, поскольку все животные, получавшие метомил (LD_{50}) в смеси в дозе 30 мг/кг массы тела, погибали. Экспериментально установлено, что доза метомила определяет токсический эффект смеси, который следует учитывать при гигиеническом регламентировании препаратов, содержащих это вещество.*

Ключевые слова: *комбинированное действие; пестицид; ксенобиотик; взаимодействие; эффект смеси*

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Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов в связи с публикацией данной статьи.

Финансирование. Исследование выполнено в рамках отраслевых программ Роспотребнадзора «Гигиеническое научное обоснование минимизации рисков здоровью населения России» (2016–2020 гг.), «Научное обоснование национальной системы обеспечения санитарно-эпидемиологического благополучия, управления рисками здоровью и повышения качества жизни населения России» (2021–2025 гг.).

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Introduction

The study of the effects of combined action (mixed effects) of xenobiotics on human health is one of the priority areas of research in the field of sanitary and hygienic (preventive) toxicology due to the need for correct assessment of the risks to public health under real exposure conditions [1–3]. One of the prominent classes of xenobiotics that has received special attention from risk-assessment experts is pesticides due to the possible negative synergistic effect of their use. The widespread use of pesticide mixtures is unavoidable due to the emergence of resistance to entire chemical classes of active substances, which is especially common for insects in relation to insecticides [4, 5].

According to the published data, the nature of the combined action of chemical mixtures may depend on the chemical family of its components, their dose in the mixture, and the target organs that its components can affect. Often, the same mixture can show multidirectional effects in relation to different studied indicators. It is known that, depending on the above factors, individual components of a mixture may exhibit either an "additive" effect or interact in such a way that their combined effects may differ from the forecasts for simple additivity. The individual components of pesticide mixtures can exhibit either "more than additive (potentiation, synergy)" or "less than the additive (inhibition, antagonism)" effect [6, 7].

The ability of mixtures of certain insecticides, particularly those that inhibit cholinesterase (organophosphorus compounds and N-methylcarbamates), pyrethroid insecticides, and triazinone fungicides, to show synergy was previously published in the scientific literature [4, 8]. At the same time, the effect of the combined action of pesticides on the body of warm-blooded animals can vary dramatically depending on the dose of each component in the mixture and on the studied indicator: at low doses, pesticides can even act as agonists for each other [8, 9].

One of the most widely used insecticidal mixtures is a combination of methomyl (CAS No. 16752-77-5 – carbamate; WHO hazard Class1b-particularly dangerous) and Bifenthrin (CAS No. 82657-04-3-pyrethroid; WHO Hazard Class II-moderate), which is applied to many plant products in agriculture in emerging economies [10, 11]. The study of the nature of the combined action of this mixture was appropriate to establish the potential danger to public health in the event of import of such products.

One of the more widely used models for studying the nature of the combined effect of chemicals in acute exposure scenarios upon ingestion *in vivo* in the late-Soviet and post-Soviet era was developed in the 1980s (hereinafter referred to as the classical model). The classical model allowed to access the combined effect of 2 chemicals in an acute experiment by employing the principles of linear regression analysis. The novelty of the approach of the classical model was in dose selection: to take into account the non-linearity of the dose-effect relationship [12, 13], the doses of the two chemicals to be administered to animals were chosen based on probabilistic values of LD_{16}, LD_{33}, and LD_{50} for each chemical.

Another convention among scientists studying the toxic effects of chemical mixtures, is that the nature of combined action can be well described by isobolograms or, if we generalize this approach to consider different dose levels, by the surface response [6, 7].

Purpose of the study. Comparative evaluation of the nature of the combined action of a mixture of insecticides methomyl and bifenthrin using various methods: using a classical model (orthogonal planning of the experiment using nonlinear variables); a generalized linear regression model for the binomial distribution (probit regression); and an isobologram.

Materials and methods

The study was conducted in the Testing Biological Laboratory of the FBIS "F.F. Erisman FSCH" of the Rospotrebnadzor according to the protocol approved by the local bioethical commission. Studies were conducted on outbred male rats. During the *in vivo* experiment, the average lethal dose of technical products and their mixtures was determined when intragastrically administered in mg/kg b.w. (LD_{50} orally). The first stage of the study included determining the average lethal dose in isolation for methomyl (CAS No. 16752-77-5) and bifenthrin (CAS No. 82657-04-3) when administered intragastrically in mg/kg b.w. The doses were selected based on the probabilistic values of LD_{16}, LD_{33} and LD_{50} of individual compounds, in accordance with the principle formulated by V.N. Rakitskii [12]. In a preliminary experiment to determine the average lethal doses for methomyl and bifenthrin, 48 male rats were used. When methomyl was administered, doses of 5, 30, 50, 60 mg/kg b.w. were tested, and when bifenthrin was administered – 30, 60, 70, 90 mg/kg b.w. (solvent – vegetable oil).

In the main experiment with a mixture of methomyl-bifenthrin, 90 outbred white male rats weighing from 215 to 230 g were used (10 animals in each group). The rats were quarantined, randomly assigned to groups, and weighed before administration. Observation of the clinical symptoms (condition and behavior of animals, reversibility of symptoms) was carried out after 30 minutes, 2 hours, 4 hours, 24 hours, after the administration, then once

Table 1 / Таблица 1

Average lethal doses (mg/kg b.w.) of methomyl and bifenthrin

Средние смертельные дозы (мг/кг массы тела) метомила и бифентрина

Dose Доза	Methomyl Метомил	Bifenthrin Бифентрин
LD_{50}	30.00	60.00
LD_{33}	23.78	30.97
LD_{17}	18.17	31.81
LD_{16}	17.79	44.72

daily for the remainder of the two weeks of the experiment. The levels of factors were chosen (LD_{16}, LD_{33}, LD_{50}) with the interval of their variation (LD_{17}). The calculation of the effect value was carried out using the formulas presented in the Methodological Recommendations "Setting up experimental studies to study the nature of the combined action of chemicals in order to develop preventive measures" [13]. The value of y as a function of (x_1, x_2) was calculated by the formula (1):

$$y = b_0 + b_1x_1 + b_2x_2 + b_{11}x_1^2 + b_{22}x_2^2 + b_{12}x_1x_2, \quad (1)$$

where y – mortality (%); x_1 – methomyl; x_2 – bifenthrin; b_0 – general direction of action with the average value of factors; b_1, b_{11} – coefficients reflecting linear and nonlinear effects of methomyl (b_2, b_{22} – bifenthrin); b_{12} – effect of combined action of methomyl and bifenthrin. The values of the coefficients b_j were calculated according to the formula (2):

$$b_j = (y_j \cdot a_j) / c_j \quad (2)$$

Additionally, the obtained data on mortality were used to construct a generalized linear regression model for the binomial distribution of the dependent variable "mortality" [14, 15]. The isobologram for observed mortality was constructed using SigmaPlot v. 12.5 (Systat Soft Wire Corporationware, USA). Statistical processing was performed in the SPSS Statistics v. 22.0 program (IBM Corporation, New York, USA) at $\alpha = 0.05$.

Results

The average lethal doses for methomyl and bifenthrin obtained in a preliminary experiment with isolated administration are presented in Table 1.

When the dose combinations (indicated in the materials and methods) were administered, the following clinical symptoms were observed in all groups: impaired coordination, tremor of the limbs and head, turning into convulsions; rapid, shallow breathing. Tremor persisted for 2–3 days after administration of the mixture and, presumably, was a manifestation of the toxic effect of bifenthrin. Death occurred within the first day after administration, mainly in the first hours. To create a mathematical model of the effect of the combination of methomyl and bifenthrin on mortality of white male rats (%) as observed in an acute oral experiment, the following doses were used (Table 2).

In Tables 3–4, we present the properties of the constructed mathematical model using the method of orthogonal planning of the experiment using nonlinear quantities (classical model) [13], which has the form (3):

$$y = 44.44 + 33.5x_1 - 5x_2 + 3.33x_1^2 + 28x_2^2 - 2.5x_1x_2, \quad (3)$$

where y is the function (x_1, x_2); x_1 – methomyl, and x_2 – bifenthrin.

It was concluded that the action of methomyl and bifenthrin exhibited interdependency ($b_{12} \neq 0$). Isolated administration of methomyl with an increase in the dose from LD_{33} to LD_{50} caused an increase in animal death by 30.17%, as evidenced by the difference in coefficients b_1–b_{11}. When combined with an increase in the dose from LD_{33} to LD_{50}, the methomyl-bifenthrin mixture caused an increase in animal death by 57.33% ($b_1 - b_2 + b_{11} + b_{12} - b_{22}$), the effect of interaction between the components of the mixture was more pronounced than the isolated effect of bifenthrin, but less than when combined with bifenthrin, as evidenced by the coefficients: $b_{12} < b_1$, $b_{12} > b_2$. The degree of action of methomyl in the studied doses was stronger than that of bifenthrin ($b_1 + b_{11} > b_2 + b_{12}$). At the same time, the leading component of the mixture was methomyl, as evidenced by the fact that the value of the coefficient b_1 exceeded the value of the coefficient b_2 ($b_1 > b_2$). Thus, the classical model indicated that the combined effects of methomyl and bifenthrin in the mixture were interdependent, but less than additive.

Then, data on animal mortality in the experiment (Table 2) were used to construct probit regressionoй model in the IBM SPSS Statistics v. 22.0 program, where the dependent variable was mortality (%), and the two independent variables were the dose levels of methomyl and bifenthrin in the mixture. The model properties are shown in Table 5: a quasi-complete separation was observed, which was caused by the fact that all animals receiving a dose of 30 mg/kg b.w. of methomyl in the mixture died, regardless of the concurrent dose of bifenthrin. After excluding doses of the mixture containing methomyl at the level of 30 mg/kg b.w. from the model, the model was reconstructed (Table 6). After studying the statistical significance of the parameters, it was concluded that when the dose of methomyl in the mixture was equal to 30 mg/kg b.w., the content of bifenthrin had no significant effect on the mortality of the mixture as all animals died. When the dose of methomyl in the mixture exceeded 30 mg/kg b.w., bifenthrin significantly affected mortality only if its amount was ⩾ 45 mg/kg b.w.

Table 2 / Таблица 2

The nature of the combined action of methomyl and bifenthrin

Характеристика совместного действия метомила и бифентрина

Experiment No. Опыт №	Probabilistic values in combination (methomyl + bifenthrin) Вероятностные значения в комбинации (метомил + бифентрин)	Dose in mg/kg b.w. Доза в мг/кг массы тела		Animal deaths (mortality / total number of animals (n)) Гибель животных (смертность / общее число животных (n))	Mortality (%) Гибель (%)
		Methomyl метомила	Bifenthrin бифентрина		
1	$LD_{16} + LD_{16}$	18	31	4/10	40
2	$LD_{16} + LD_{33}$	18	45	10/10	10
3	$LD_{16} + LD_{50}$	18	60	5/10	50
4	$LD_{33} + LD_{16}$	24	31	10/10	100
5	$LD_{33} + LD_{33}$	24	45	3/10	30
6	$LD_{33} + LD_{50}$	24	60	6/10	60
7	$LD_{50} + LD_{16}$	30	31	10/10	100
8	$LD_{50} + LD_{33}$	30	45	10/10	100
9	$LD_{50} + LD_{50}$	30	60	10/10	100

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Table 3 / Таблица 3

Experimental plan for the study of acute combined exposure to two factors (x_1, x_2)

Экспериментальный план по изучению острого комбинированного воздействия двух факторов (x_1, x_2)

Experiment No. Опыт №	x_1	x_2	Actual mortality (%) Фактическая смертность (%) y_j	Estimated mortality (%) Предполагаемая смертность (%) y_{pj}	Error Ошибка $j = y_j - y_{pj}$
1	−1	−1	40	45.28	−5.28
2	1	−1	100	116.94	−16.94
3	−1	1	50	40.28	9.72
4	1	1	100	101.94	−1.94
5	0	0	30	44.44	−14.44
6	0	1	60	67.78	−7.78
7	0	−1	100	77.78	22.22
8	1	0	100	81.11	18.89
9	−1	0	10	14.44	−4.44

Table 4 / Таблица 4

Vectors for calculating the coefficients $b_j = (y_j \cdot a_j) / c_j$ for $y = f(x_1, x_2)$

Векторы для расчёта коэффициентов $b_j = (y_j \cdot a_j) / c_j$ для $y = f(x_1, x_2)$

C_j	9	6	6	6	6	4
	a_0	a_1	a_2	a_{11}	a_{22}	a_{12}
Experiment No. Опыт №						
1	−1	−1	−1	1	1	1
2	−1	1	−1	1	1	−1
3	−1	−1	1	1	1	−1
4	−1	1	1	1	1	1
5	5	0	0	−2	−2	0
6	2	0	1	−2	1	0
7	2	0	−1	−2	1	0
8	2	1	0	1	−2	0
9	2	−1	0	1	−2	0

Table 5 / Таблица 5

Parameters of the GLM model in IBM SPSS Statistics v. 24 with quasi-complete separation in relation to the absolute mortality of methomyl at the dose of 30 milligrams per kilogram of animal body weight

Параметры модели GLM в IBM SPSS Statistics v. 24 с квазиполным разделением в отношении абсолютной смертности от метомила в дозе 30 миллиграммов на килограмм массы тела животного

Parameter estimation Parameter Оценка параметра Параметр	B	Standard Error Стандартная ошибка	95% Wald Confidence Interval 95%-й доверительный интервал Вальда		Hypothesis testing Проверка гипотезы			Exponent (B) Экспонента (B)	95% Wald Confidence Interval for Exponent (B) 95%-й доверительный интервал Вальда для Экспоненты (B)	
			lower limit нижний предел	upper limit верхний предел	chi-squared Wald χ^2 Вальда	degrees of freedom степени свободы	statistical significance статистическая значимость p		lower limit нижний предел	upper limit верхний предел
(Intersection) / (Пересечение)	−7.411	4707.8581	−9234.644	9219.821	0.000	1	0.999	0.001	0.000	.[a]
[Methomyl = 18 mg/kg b.w.] [Метомил = 18 мг/кг м.т.]	7.750	4707.8581	−9219.483	9234.982	0.000	1	0.999	2320.993	0.000	.[a]
[Methomyl = 24 mg/kg of b.w.] [Метомил = 24 мг/кг м.т.]	6.804	4707.8581	−9220.428	9234.036	0.000	1	0.999	901.532	0.000	.[a]
[Methomyl = 30 mg/kg b.w.] [Метомил = 30 мг/кг м.т.]	0[b]	.	.	.	.	.	.	1	.	.
[Bifenthrin = 31 mg/kg b.w.] [Бифентрин = 31 мг/кг м.т.]	−0.486	0.4313	−1.332	0.359	1.272	1	0.259	0.615	0.264	1.432
[Bifenthrin = 45 mg/kg b.w.] [Бифентрин = 45 мг/кг м.т.]	1.064	0.4441	0.193	1.934	5.737	1	0.017	2.897	1.213	6.917
[Bifenthrin = 60 mg/kg b.w.] [Бифентрин = 60 мг/кг м.т.]	0[b]	.	.	.	.	.	.	1	.	.
(Scale) / (Шкала)	1[c]	–	–	–	–	–	–	–	–	–

N o t e: Here and in Table 6: Dependent variable: Mortality Model: (Intersection), Methomyl, Bifenthrin. a. Set to "System unavailable due to overflow". b. Set to zero because this parameter is redundant. c. Fixed to the displayed value.

П р и м е ч а н и е. Здесь и в табл. 6: Зависимая переменная: Модель смертности: (пересечение), Метомил, бифентрин. a – Установлено значение «Система недоступна из-за переполнения». b – Установлено нулевое значение, поскольку этот параметр является избыточным. c – Зафиксировано на отображаемом значении.

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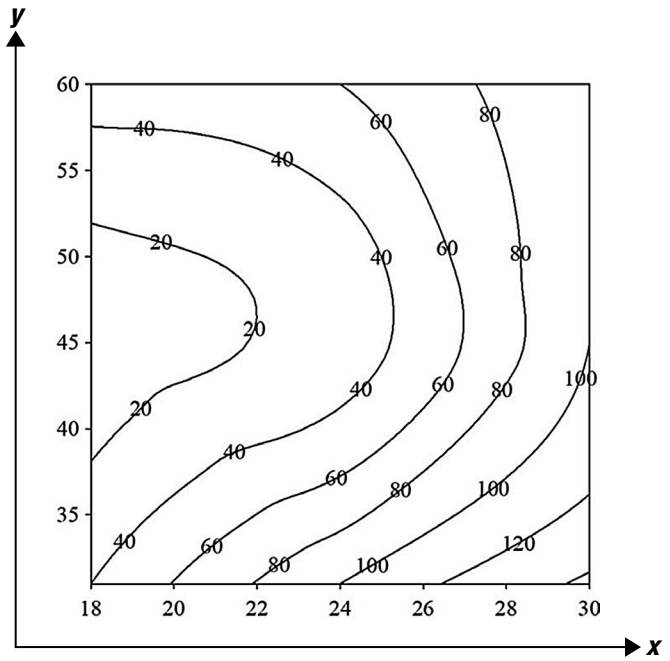
Table 6 / Таблица 6

Parameters of the GLM model in IBM SPSS Statistics v. 24, constructed by excluding the dose of methomyl of 30 milligrams per kilogram of animal body weight due to quasi-complete separation caused by absolute mortality

Параметры модели GLM в IBM SPSS Statistics v. 24, построенные путем исключения дозы метомила 30 миллиграмм на килограмм массы тела животного в связи с квазиполным разделением, вызванным абсолютной смертностью

Parameter estimation Parameter Оценка параметра Параметр	B	Standard Error Стандартная ошибка	95% Wald Confidence Interval 95%-й доверительный интервал Вальда		Hypothesis testing Проверка гипотезы			Exponent (B) Экспонента (B)	95% Wald Confidence Interval for Exponent (B) 95%-й доверительный интервал Вальда для Экспоненты (B)	
			lower limit нижний предел	upper limit верхний предел	chi-squared Wald χ^2 Вальда	degrees of freedom степени свободы	statistical significance статистическая значимость *p*		lower limit нижний предел	upper limit верхний предел
(Intersection) / (Пересечение)	–0.607	0.3441	–1.282	0.067	3.113	1	0.078	0.545	0.278	1.070
[Methomyl = 18 mg/kg b.w.] [Метомил = 18 мг/кг м.т.]	0.946	0.3696	0.221	1.670	6.545	1	0.011	2.574	1.248	5.313
[Methomyl = 24 mg/kg of b.w.] [Метомил = 24 мг/кг м.т.]	0[a]	.	.	.	.	.	.	1	.	.
[Bifenthrin = 31 mg/kg b.w.] [Бифентрин = 31 мг/кг м.т.]	–0.486	0.4313	–1.332	0.359	1.272	1	0.259	0.615	0.264	1.432
[Bifenthrin = 45 mg/kg b.w.] [Бифентрин = 45 мг/кг м.т.]	1.064	0.4441	0.193	1.934	5.737	1	0.017	2.897	1.213	6.917
[Bifenthrin = 60 mg/kg b.w.] [Бифентрин = 60 мг/кг м.т.]	0[a]	.	.	.	.	.	.	1	.	.
(Scale) / (Шкала)	1[b]	–	–	–	–	–	–	–	–	–



The isobologram of the combined action of methomyl and bifenthrin. Vertical axis (*y*): dose of bifenthrin in the mixture in milligrams per kilogram of animal body weight. Horizontal axis (*x*): dose of methomyl in the mixture in milligrams per kilogram of animal body weight. The lines show the change in mortality rate as a percentage.

Изоболограмма комбинированного действия метомила и бифентрина. Вертикальная ось (*y*): доза бифентрина в смеси в миллиграммах на килограмм массы тела животного. Горизонтальная ось (*x*): доза метомила в смеси в миллиграммах на килограмм массы тела животного. Линии показывают изменение показателя смертности в процентах.

The construction of an isobologram (Figure) confirmed that methomyl was the most significant component in the mixture [16]. At doses of methomyl ⩾ 24 mg/kg b.w., the predicted mortality of the mixture was ⩾ 100% regardless of the dose of bifenthrin.

Discussion

Plant protection products based on methomyl have been used worldwide since 1978 [17]. In 2018, more than 100 thousand kilograms of methomyl-based formulations were used in California, USA alone [18]. Methomyl is often used in combination with bifenthrin in the form of mixed formulations due to the need to counteract acquired resistance to carbamates in target organisms (insects) [10, 11]. Both active substances affect the nervous system: the principle of action of methomyl is to inhibit the enzyme acetylcholinesterase, while bifenthrin disrupts the normal functioning of the sodium channels of neurons, interfering with their closure [19]. In mammals, poisoning with these active substances causes hyperstimulation of the nervous system, which is manifested by similar clinical symptoms, such as convulsions, gait disorders, etc. [20–22]. However, the data obtained in the present study demonstrates that, despite the similarity of the mechanisms of toxic action of these substances, the content of methomyl in the mixture, due to its higher toxicity when administered orally, is the main predictor of mortality. Additive effect was shown only at those doses of the mixture when the individual dose of methomyl was less than 30 milligrams per kilogram of animal body weight, which corresponds to the expected LD_{50} for methomyl. Thus, when using methomyl- containing combinations, it is necessary to pay special attention to the hygienic regulation of their use and the use of personal protective equipment due to the high toxicity of methomyl.

Conclusion

In the present study the dose of methomyl dictated the toxic effect of the mixture (with bifenthrin). This “dominance” of methomyl should be accounted for in hygienic regulation of methomyl-containing formulations. Furthermore, the present study demonstrates the importance of assessing mixture-based pesticide interactions for human risk assessment.

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(п.п. 1–5, 7–11, 15–22 см. References)

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