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An oral developmental toxicity study of generic pesticide pinoxaden in rabbits

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ABSTRACT

The safety assessment of pinoxaden by the Joint Meeting on Pesticide Residues (JMPR) established a NOAEL of 30 mg/kg bw/day for maternal and embryo/fetal toxicity from a rabbit developmental toxicity study. However, the Pesticide Peer Review Expert meeting (EFSA) lowered the NOAEL to 10 mg/kg bw/day due to observed diaphragm malformations in one developmental toxicity study in rabbits, proposing a classification for developmental effects as Category 2 R63 or H361d. Both JMPR and EFSA set the Acceptable Daily Intake (ADI) at 0.1 mg/kg bw/day, derived from a 2-year rat study NOAEL with a safety factor of 100, but EFSA also supported ADI by teratology study in rabbits. The current prenatal developmental toxicity study on pinoxaden aimed to elucidate and clarify the potential teratogenic effects and could provide supplementary data for determining the ADI for pinoxaden. The study design exceeded the OECD TG 414 by including an assessment of internal organs. The test item was orally administered by gavage daily from day 6 to day 28 of gestation to three groups of animals, each composed of 21 females, in dose levels of 0, 10 and 30 mg/kg/bw/day. One female from the 30 mg/kg/bw/day dose group was euthanized in extremis on Day 27 *post-coitum* due to premature delivery, likely induced by poor general condition and was therefore considered to be an indirect effect of the test item. One female at 30 mg/kg/bw/day had entirely dead litters except for one live male pup (9 non-live implants vs 1 live fetus). Since the incidence of post-implantation loss or mean number of the dead pups within the remaining dams at 30 mg/kg/ bw/day that survived to necropsy was not significantly increased, we assume that the toxic effect was on the dam, rather than on the conceptus. No pinoxaden-related skeletal or visceral variations or malformations were observed. No evidence of developmental toxicity was observed. Under the conditions of the study, the pinoxaden produced maternal toxicity at a high dose tested; thus, NOAEL for maternal toxicity was determined to be 10 mg/kg bw/day. NOAEL for developmental toxicity was established at 30 mg/kg bw/day. The obtained results may supplement the overall safety and toxicity profile of pinoxaden. Nevertheless, the NOAEL determined in this study does not affect the previously established ADI.

1. Introduction

Pinoxaden is a selective phenylpyrazolin pesticide (herbicide) widely used in agriculture in European Union countries and Ukraine to control grass weeds in cereal fields [1]. To date, published studies on pinoxaden have predominantly concentrated on its mechanism of action, exploration of resistance mechanisms, efficacy in the application, and residue analysis [2], [3], [4], [5], [6], [7]. The toxicity properties of pinoxaden on mammals were published in reports by several regulatory authorities (JMPR, 2005; EFSA, 2013) [8], [9]. According to these reports [8], [9], pinoxaden is a non-genotoxic compound and does not show acute toxicity properties via dermal or oral routes but was mentioned as "*may act as a skin sensitizer, a skin, eye, and respiratory tract irritant*". Pinoxaden demonstrates no potential for carcinogenicity, neurotoxicity or reproductive toxicity. Treatment with pinoxaden led to kidney injuries in male rats and induced changes in their water consumption and urine volume during a 2-year chronic toxicity study, with the relevant systemic No Observed Adverse Effect Level (NOAEL) identified as

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10 mg/kg/bw/day. Several developmental toxicity studies [8], [9] were conducted on pinoxaden in different rodent species (rats and rabbits) to evaluate its teratogenic properties.

In a developmental toxicity study involving rats administered orally pinoxaden during gestation at various doses (0, 3, 30, 300 and 800 mg/kg bw/day), maternal toxicity was observed at doses of 300 mg/kg bw/day or higher, characterized by reduced body weight gain and feed consumption. Embryo and fetal toxicity were noted at the same dose levels, manifested by incomplete ossification [8], [9].

Two developmental toxicity studies of pinoxaden on rabbits were performed using the same dose levels of 0, 3, 10, 30 and 100 mg/kg bw/day [8], [9]. At a dosage of 100 mg/kg bw/day in the first developmental toxicity study on rabbits [8], [9], there was a slight rise in the occurrence of diaphragm defects, with hernias observed in two fetuses/litters and a fissure noted in one fetus/litter. Also, diaphragm malformation was observed in 1 fetus at a dose level of 30 mg/kg bw/day [9]. In a second guideline developmental toxicity study on rabbits, at a dose of 100 mg/kg bw/day of pinoxaden, maternal animals' decreased body weight, feed consumption, and total litter loss were observed [8], [9].

Additionally, two nonstandard (not fully covered by the OECD TG 414 requirements for studies) developmental toxicity studies of pinoxaden at a dose of 100 mg/kg bw/day were performed to investigate the potential genetic influence on diaphragm defects in rabbits, but the results did not reveal consistent findings [8].

The Joint Meeting on Pesticide Residues (JMPR) concluded that teratogenic findings in one rabbit developmental toxicity study out of four are incidental and established NOAEL for pinoxaden of 30 mg/kg/bw/day for maternal and developmental toxicity. Pinoxaden was concluded not to be teratogenic [8].

Despite this, the European Food Safety Authority (EFSA) considered: *"a low incidence of diaphragm malformations in one developmental toxicity study in rabbits as a reason for lowering NOAEL to 10 mg/kg/bw/day, along with a proposed classification for developmental effects (Cat 2 R63 "Possible risk of harm to the unborn child", or H361d: Suspected of damaging the unborn child)"* [9].

EFSA and JMPR established Acceptable Daily Intake (ADI) at level 0.1 mg/kg bw/day, based on a NOAEL of 10 mg/kg bw/day from a 2-year toxicity and carcinogenicity study on rats using a safety factor of 100 [8], [9], also, EFSA mentioned that deriving the ADI was supported by teratology study on rabbits [9].

Given that generic pesticide technical products are produced by different manufacturers and the purity of generic pinoxaden 98.1 % differs from the original molecule (with a specified minimum purity of 97 % for the active substance pinoxaden [9]), which was evaluated for developmental endpoints, impurities or a higher concentration of the active substance could impact the observed effects. Thus, the current study on the generic pesticide pinoxaden aimed to evaluate the equivalence to the original molecule of pinoxaden and elucidate and clarify its potential teratogenic effects on rabbits under Good Laboratory Practice (GLP) conditions.

2. Material and methods

The study was performed in the GLP-certified laboratory of experimental toxicology and mutagenesis, L.I. Medved's Research Centre of Preventive Toxicology, Food and Chemical Safety, Ministry of Health, Ukraine. The study was conducted following the requirements and provisions of the Commission for the Ethics of Medical and Biological Research of L.I. Medved's Research Centre of Preventive Toxicology, Food and Chemical Safety, Ministry of Health, Ukraine (Law of Ukraine No. 3447-IV of February 21, 2006 "On the Protection of Animals from Cruelty") and European Communities Council Directive of November 24, 1986 (86/609/EEC).

The present study was conducted in New Zealand albino rabbits by oral exposure to pinoxaden according to modified OECD TG 414 (Prenatal Developmental Toxicity Study) [10]. The study design exceeded the OECD TG 414 by including a liver and paired kidney weight assessment. While the two doses (vs. three, as recommended by OECD TG 414) were used, adhering to the 3Rs and bioethics principles.

2.1. Identification of test item

The test item, herbicide pinoxaden technical (generic formulation), CAS number 243973–20–8, was 98.1 % pure powder. Dosing formulation solutions were prepared daily by individually weighing the appropriate amount of pinoxaden and dissolving it in distilled water with a few drops of an emulsifier (OP-10) as a solvent.

2.2. Doses selection

The prenatal developmental toxicity study protocol [10] requires at least three dose levels and a concurrent control group. This study has been designed for the generic pinoxaden; thus, adhering to the principles of the 3Rs and authorities' published data [9], [8], the two dose levels of 10 and 30 mg/kg/ bw/day and a concurrent control group were selected (see Table 1 below).

2.3. Experimental animals

The 63 inseminated New Zealand albino rabbits were received in good health from the Limited Liability Company "Dali-2000" in Kyiv, Ukraine and acclimated for 6 days before the pinoxaden administration. Veterinary care was available throughout the acclimatization period and the course of the study.

2.4. Animal husbandry and diet

Rabbits were housed under standard laboratory conditions in an environmentally monitored air-conditioned room with adequate fresh air supply (12 volumes per hour) by prepared clean HEPA-filter air, room temperature 17°C to 19°C and relative humidity 45–65 % with 12 hours of light and 12 hours of dark cycle. The temperature and relative humidity were recorded twice daily during the acclimatization and experimental period. Rabbits were individually housed in metal cages with mesh bottoms and areas of shelter and height for exploration. The cages were cleaned and disinfected weekly. A pellet diet (PK 92–1) for rabbits produced by PE "Rezon-1" (Ukraine), free of substances which could compromise the outcome of the study, was used. Water passed through a reverse osmosis unit, and disinfected ultraviolet radiation was provided in glass water bottles with stainless steel sipper tubes. Food and water were provided *ad libitum* throughout the study period.

2.5. Group formation and administration

The females were allocated to experimental groups, each consisting of 21 rabbits, and randomization was based on their body weight. On day 6 of gestation (GD 6), body weight values ranged from 2400 g to 3710 g.

The test item and vehicle were administered daily by oral gavage 7 days a week from GD 6 (considered as implantation day) to GD 28 [10]. The selected route of administration was oral since this is a potential route of human exposure. Females were exposed in the morning at approximately the same time each day. Individual dosages were based on the most recently recorded body weight to provide the correct mg/kg/bw/day dose. The concurrent control group of females received the vehicle. Tested substances were administered using a plastic catheter attached to a plastic disposable syringe. The dosing formulations were stirred continuously during exposure. Any residual dosing volumes after exposure were discarded. Table 1 displays the group formation and corresponding doses, along with the concentrations at which the animals were administered.

Table 1
Group formation and administration of female rabbits.

Group No	Group Identification	Test Item Identification	Dose Level (mg/kg bw/day)	Dose Volume (mL/kg)	Dose Concentration (mg/mL)	Number of Females
1	Control	Distillated water with an emulsifier	0	5	0	21
2	Low dose	Pinoxaden	10	5	2	21
3	High dose	Pinoxaden	30	5	6	21

2.6. Study design

2.6.1. Mortality and clinical observation

All rabbits were monitored for morbidity and mortality throughout the study. Each female was observed for signs of toxicity during the dosing, at 30 minutes, 1- and 4-hour after dose administration. Animals were removed from the home cage during observations only in case of identification or confirmation of possible findings. In addition, each cage debris was examined to detect premature birth or abortion. Animals showing premature birth or abortion were sacrificed *in extremis*.

2.6.2. Body weight

Individual female body weights were recorded on the day of insemination (GD 0) and group assignment (GD 6) and at three-day intervals thereafter (GD 9, 12, 15, 18, 21, 24, and 28). Body weight value on GD 28 (i.e. the day of scheduled necropsy) was corrected for gravid uterine weight to calculate maternal body weight change.

2.6.3. Food consumption

Food consumption was quantitatively measured at three-day intervals starting from GD 6 until the completion of the in-life part of the study.

2.6.4. Euthanasia

Animals surviving until the scheduled necropsy were euthanized by CO_2 asphyxiation on GD 28. Females with premature birth or abortion were euthanized within 24 hours after detection.

2.6.5. Necropsy of females and examination of uterine content

A complete autopsy was conducted for all females who died spontaneously, euthanized *in extremis*, or at scheduled termination. Necropsy included a macroscopical examination of the external surface of the body, all orifices, the cranial cavity, and the external surface of the abdominal and pelvic cavities, including viscera. The liver, kidney, and gravid uterus weight was recorded for each dam (with at least one live fetus).

Each ovary and uterine horn of dams were examined to determine:
- the number of *corpora lutea*;
- the number of implantation sites;
- the number of early or late resorptions;
- the number of live or dead/recently dead fetuses.

Early resorption was defined as one in which organogenesis was not grossly evident; late resorption was defined as one in which the occurrence of organogenesis and autolysis was grossly evident. The total number of implantation sites was calculated as the sum of the number of live or dead/recently dead fetuses and early or late resorptions in each dam [11]. Dead fetuses were not examined for visceral or skeletal anomalies if autolysis of the body was noted.

2.6.6. Fetal examination

After a cesarean section, fetuses with their placentas were removed in serial order, blotted on a moist surface, freed of adherent material, exanimated for gross external abnormalities, sexed, and weighed. Recently, dead fetuses were sent for visceral examination. Dead fetuses were discarded.

The fetal findings were described according to the harmonized terminology of the International Federation of Teratology Societies (IFTS) [12] without categorization and were classified as variations or malformations: variation (minor abnormality) refers to structural change considered to have no detrimental effect on the animal or have low effect and may occur relatively frequently in the control population (transient); malformation (major abnormality) refers to structural change considered detrimental or was incompatible with life to the animal and occur usually rare.

2.6.7. Soft tissues examination

The sex of each fetus was confirmed by visceral examination of the gonads. Fetuses were evaluated for visceral anomalies during a scheduled necropsy. The heads were removed from approximately one-third of the fetuses in each litter and placed in Bouin's fluid for subsequent soft-tissue examination using the Wilson sectioning technique [13].

2.6.8. Skeletal examination

All fetuses were eviscerated, followed by fixation with 96 % ethanol, macerated with KOH, stained with Alizarin Red S, and cleared with aqueous glycerol solutions using the Dawson technique [14]. Each skeleton was examined for the presence of abnormalities. The skull was examined for degree of ossification of the nasal, parietal, interparietal, supraoccipital, exoccipital, lacrimal, zygomatic, squamosal, premaxillary, maxillary, basisphenoid, hyoid, and tympanic ring. The vertebral and sterna centers were counted for the number of ossification centers. The number of ribs was counted, and their shape was evaluated. The cervical, thoracic, lumbar, sacral, and caudal vertebrae were examined for the ossification of centers. Pelvic girdles, forelimbs, and hindlimbs were observed for bone development.

2.7. Statistical analysis

Data analysis was conducted using GraphPad Prism (version 8, GraphPad). Group mean values, standard deviations (SD), and standard error of the mean (SEM) were calculated where applicable (results presented as mean ± SEM in tables). Statistical significance was set at $p \leq 0.05$ for all analyses. Group comparisons were assessed using a one-way analysis of variance (ANOVA) followed by Dunnett's test for treated versus control groups. Fetal alterations were evaluated using the Fisher Exact test. Fetal sex ratios were analyzed using the Chi-square test.

The following constructed variables were calculated:

- Preimplantation loss = ((No. corpora lutea - No. of implantations) / No. corpora lutea) × 100;
- Postimplantation loss = ((No. of implantations - No. of live fetuses) / No. of implantations) × 100;
- % fetuses with abnormalities in the litter = (No. of live fetuses with abnormalities in a litter / No. of overall live fetuses) × 100;
- % fetuses with abnormalities in the group = (Σ (% fetuses with abnormalities in all litters) / No. of litters in the group) × 100;
- % litters with abnormalities = (Σ (of all litters in a group with fetal abnormalities) / No. of litters in the group) × 100;
- Sex Ratio (% males)

3. Results

3.1. Mortality and clinical observation

No abnormal clinical signs were observed during the acclimatization period.

Two females died during the treatment period before the scheduled necropsy – one in the control group and one at the 10 mg/kg bw/day dose. Before death, these females had laboured breathing or abnormal vocalization. The clinical observations and pathology findings indicated a dosing error or mechanical injury and, thus, were considered not to be pinoxaden-related.

The pregnancy rates were 95.24 %, 90.48 %, and 95.24 % in the control, low dose and high dose, respectively, which was considered as normal in this strain and age of rabbits [15].

No premature births in the control or at 10 mg/kg/ bw/day were noted. However, one female treated with 30 mg/kg/ bw/day was sacrificed *in extremis* on GD 27 after starting to deliver offspring. Clinical observations revealed erected fur and body weight loss (up to 7 % compared to their maximum body weight) during the treatment period before premature birth. Macroscopical evaluation of its fetuses did not reveal any external abnormalities.

We observed a single occurrence of diarrhea in the absence of a dose-response, which might be attributed to stress from handling [16] rather than an effect of pinoxaden treatment. Any other clinical signs noted during the treatment period occurred within the range of background findings to be expected for rabbits of this age and strain [17], [18].

3.2. Body weight and food consumption

The maternal mean body weights and body weight gain of treated animals remained in the same range as controls over the treatment period. The maternal mean food consumption was similar to the control level over the study period (Table 2)

3.3. Necropsy of females and examination of uterine content

The total number of pregnant rabbits available for evaluation at scheduled necropsy was 19, 19, and 18 for the control, low and high dose groups, respectively. No treatment-related effects were observed on the maternal liver, paired kidney, and gravid uterine relative or absolute weights. The weight corrected for gravid uterus, numbers of *corpora lutea*, implantation sites, and pre-and postimplantation sites in the control and test groups were similar and within the range of normal biological variation (Table 3) [19].

One female at 30 mg/kg/bw/day had entirely dead litters except for one live male fetus (9 non-live implants (6 dead fetuses, and 3 late resorptions) vs 1 live fetus). One live male fetus from this litter was small for gestation age and was sent for visceral examination.

3.4. Examination of fetuses

The absolute live litter size was 152, 151, and 139 pups in the control, low, and high dose groups, respectively. The mean live litter size was unaffected by treatment with pinoxaden. Differences from the control group were not statistically significant.

No test item-related effects on external abnormalities were noted. A rabbit fetus was judged as small for gestational age when its size was below the normal range of variation of its littermates [20]. One fetus at 10 mg/kg bw/day was small for gestational age, but since it was isolated occurring, it was considered an incidental finding.

No test item-related effects on fetal body weights or placenta weights (both sexes) were noted at any dose level. The male-to-female ratio was unaffected by treatment with pinoxaden. Dead fetuses were judged as sex undetermined due to the autolysis of the bodies.

In total, seven dead fetuses at 30 mg/kg bw/day (from different litters), one fetus at 10 mg/kg bw/day, and two in the control group, respectively. Differences were not statistically significant compared to the concurrent control value (Table 4).

3.5. Soft tissues examination

No pinoxaden-related visceral variations or malformations were observed. Visceral findings were observed in 2, 1, and 1 fetuses from the control, low, and high dose groups, respectively. Visceral variations - incomplete lobulation of the lungs was noted for two control fetuses from one litter and one fetus at 10 mg/kg bw/day. The high-dose fetus

Table 2
Body weight changes and food consumption of female rabbits during the gestation period.

Parameters	Control Group	Treated Groups	
		10 mg/kg/ bw/day	30 mg/kg/ bw/day
Number of pregnant females	19	19	18[a]
Body weight, g			
Gestation day 0	2816.32 ± 65.25	2831.94 ± 53.44	2855.00 ± 63.99
Gestation day 6	2911.68 ± 78.52	2930.67 ± 66.09	2962.78 ± 65.92
Gestation day 28	3329.44 ± 81.95	3318.24 ± 64.73	3358.00 ± 74.45
Body weight gain, g			
Gestation days 0–6 (pre-exposure period)	95.37 ± 25.38	98.72 ± 33.33	107.78 ± 48.53
Gestation days 6–28 (exposure period)	349.72 ± 26.37	335.88 ± 38.23	346.88 ± 35.50
Food consumption, g			
Gestation days 0–6 (pre-exposure period)	1084.06 ± 76.77	1218.81 ± 53.82	1185.00 ± 47.64
Gestation days 6–28 (exposure period)	2460.72 ± 213.36	2652.38 ± 137.07	2522.24 ± 172.57

Results presented as Mean ± SEM.
[a] Value was obtained from 18 pregnant rabbits till gestation day 24.

Table 3
Reproductive and developmental parameters in rabbits on Day 28 of pregnancy after exposure to pinoxaden.

Parameters	Control Group	Treated Groups	
		10 mg/kg/ bw/day	30 mg/kg/ bw/day
Number of inseminated females	21	21	21
Number of females with premature birth	0	0	1
Number of pregnant females	19	19	18
Number of females with live fetuses	19	19	17
Number of *corpora lutea*	10.06 ± 0.42	9.78 ± 0.38	10.56 ± 0.47
Number of live fetuses (total)	8.44 ± 0.38	8.39 ± 0.59	8.69 ± 0.78
Number of live male fetuses	4.28 ± 0.39	3.89 ± 0.38	4.50 ± 0.49
Number of live female fetuses	4.17 ± 0.33	4.50 ± 0.32	4.19 ± 0.53
Number of dead fetuses	0.11 ± 0.11	0.06 ± 0.06	0.44 ± 0.38
Average live fetus weight (g)	41.13 ± 0.87	41.87 ± 2.03	40.46 ± 1.05[a]
Average live fetus placenta weight (g)	6.59 ± 0.13	6.42 ± 0.12	6.57 ± 0.10[a]
Pre-implantation loss (%)[b]	10.44 ± 3.60	9.57 ± 3.16	6.37 ± 2.71
Post-implantation loss (%)[c]	3.90 ± 1.65	4.54 ± 2.32	3.92 ± 1.32

g = grams; % = percentage.
Results presented as Mean ± SEM.
[a] Value was obtained from 17 pregnant rabbits.
[b] (No. of preimplantation embryonic loss/no. of corpora lutea)x100.
[c] (No. of resorptions and dead fetuses/no. implantations)x100.

Table 4
Fetal external examinations.

Parameters	Control Group	Treated Groups	
		10 mg/kg/ bw/day	30 mg/kg/ bw/day
Total number of fetuses (litters) examined	152(19)	151(19)	139(17)
Total number of dead fetuses (litters)	2(1)	1(1)	7(2)
Total number of fetuses (litters) with external abnormalities	0(0)	1(1)	0(0)
Total number of fetuses (litters) small for gestational age	0(0)	1(1)	0(0)

Statistics were compared against the control group.

had a malpositioned adrenal gland (one live male pup from litter with entirely dead litters). The incidental occurrence and distribution among groups of these findings did not suggest a test item-related effect; therefore, they were considered to have occurred by chance (Table 5).

3.6. Skeletal examination

No pinoxaden-related skeletal variations or malformations were observed. There were 7, 9, and 4 fetuses with skeletal findings in the control, low, and high-dose groups, respectively. Skeletal variations - incomplete ossification or dislocation of sternebrae was noted for six control fetuses from three litters, nine fetuses from five litters at 10 mg/kg bw/day, and three fetuses from two litters at 30 mg/kg bw/day. This group distribution did not indicate any test item relationship. The remaining findings included wavy ribs (one fetus from one litter; high-dose group) and short cervical ribs (one fetus from one litter; control group), which occurred singly and were within historical range and, therefore, were considered chance findings. All skeletal variations occurred without a dose-dependent manner trend and/or infrequently; thus, they were considered not test item related (Tables 5 and 6).

4. Discussion

Previously reported developmental toxicity studies [8], [9] of pinoxaden in rabbits supported the establishment of ADI. Our study was focused on determining the effects of pinoxaden on the developmental organism after maternal exposure during the critical period of organogenesis and evaluating maternal toxicity under GLP conditions with additional design elements (i.e. internal organs assessment) considered to evaluate additional endpoints of possible maternal toxicity of pinoxaden [8], [9].

The New Zealand albino rabbits were chosen as the animal model for the study as this species was used in previous studies published by regulatory agencies [10], [21]. Moreover, the New Zealand albino rabbits were selected for this study because they have been used extensively for developmental studies at the testing laboratory and historical control data are available. Acceptable models for assessing the teratogenic potential of chemicals that do not use live laboratory animals currently do not exist [22]. The total number of animals used in this study was considered the minimum required to characterize the developmental toxicity potential of the pinoxaden property.

Table 5
Soft tissue examinations of fetuses.

Parameters	Control Group	Treated Groups	
		10 mg/kg/ bw/day	30 mg/kg/ bw/day
Total number of fetuses examined[a,b]	50	50	44
Total number of fetuses (litters) with visceral findings	2(1)	1(1)	1(1)
Incomplete lobulation of the lungs	2(1)	1(1)	0(0)
Malpositioned adrenal gland	0(0)	0(0)	1(1)

Statistics were compared against the control group.
[a] Dead fetuses were not exanimated due to autolysis of the body.
[b] All fetuses, except the dead ones, were examined for visceral anomalies. The heads were examined of one-third of the fetuses in each litter

Table 6
Skeletal examinations of fetuses.

Parameters	Control Group	Treated Groups	
		10 mg/kg/ bw/day	30 mg/kg/ bw/day
Total number of fetuses examined[a,b]	100	100	88
Total number of fetuses (litters) with skeletal findings	7[c](4)[d]	9(5)	4(3)
Sternebrae:			
Incomplete ossification	5(2)	6(3)	3(2)
Dislocation	1(1)	3(2)	0(0)
Rib(s):			
Wavy	0(0)	0(0)	1(1)
Short cervical ribs	1(1)	0(0)	0(0)

Statistics were compared against the control group.
[a] Dead fetuses were not exanimated due to autolysis of the body.
[b] All fetuses, except the dead ones, were examined for skeletal anomalies. Two-thirds of the fetuses in each litter were examined for skeletal anomalies without heads.
[c] Number of fetuses with findings. [d] Number of litters in which findings were observed.

4.1. Details on maternal toxic effects

Treatment-related maternal effects in the present study were limited to one incidence of premature birth at the highest dose tested. Premature birth is defined as the expulsion of conceptuses on day 27 of gestation or earlier [23]. In our opinion, the premature birth was most likely caused by female's poor general condition and was, therefore, considered to be an indirect effect of pinoxaden treatment.

While it is normal to expect a small number of post-implantation losses within individual litter within a study, the presence of total litter loss is a rare event. Indeed, the presence of more than one dam/group with total litter loss is sporadic. Within the classification guidance documents [24], [25], this endpoint is discussed as "pregnancy outcomes", "fertility", or "death of the developing organism", and as such, can be considered either a reproductive or developmental outcome. Under both scenarios, the loss of implantations during pregnancy is regarded as a severe outcome.

According to guidance documents [25], [26], survival endpoints can be affected by the toxicity of a test item, either by direct effects on the offspring or indirectly through effects on the ability of the dam to support the offspring. This statement is in line with the Khera [27] publication, which reported that only nine chemicals (four each in hamsters and rabbits and one in rats) were noted to induce embryo-fetal deaths at apparently maternal non-toxic doses. These findings tend to suggest a contributory role for maternal toxicity in the induction of embryo-fetal deaths. Presumptive evidence indicates that embryo-fetal deaths, which in published literature are presently attributed to chemical induction for a large number of chemicals, may be a consequence of maternal toxicity per se [27], [28].

In this study, one female at 30 mg/kg/bw/day had entirely dead litters except for one live male pup (6 non-live fetuses vs 1 live fetus), indicating an 85.7 % incidence of fetal mortality per this litter (where the percentage of fetal mortality/litter in the control group is 0.95 and in high dose group – 6.4 %). Since the incidence of post-implantation loss or mean number of the dead pups within the remaining dams at 30 mg/kg/ bw/day that survived to necropsy was not significantly increased, we assume that the toxic effect was most likely on the dam, rather than

on the conceptus.

According to classification guidance documents [24], [25], maternal toxicity can be defined as any adverse effect in the dam. Still, the only maternal endpoints routinely evaluated in a prenatal developmental toxicity study are clinical signs, body weight change, food intake and macroscopic findings at necropsy. However, the rabbit species has been relatively understudied [29], and each maternal toxicity endpoint should be evaluated in detail separately [30,31]. This study showed no significant changes between control and treatment groups in the mentioned maternal endpoints. In our opinion, one finding of premature birth and one finding of entirely dead litters except for one live male pup (9 non-live fetuses vs 1 live fetus) without similar findings in the concurrent control group should be extrapolated as a direct effect of pinoxaden treatment on dams. Thus, the highest dose tested (30 mg/kg/bw/day) may be considered for maternal toxicity as the lowest-observed-adverse-effect level (LOAEL).

4.2. Details on fetal teratogenic and developmental effects

No test item-related changes were noted in any of the developmental parameters. None of the fetuses showed any pinoxaden-related skeletal or visceral abnormalities at any dosage group. All findings were isolated, distributed randomly amongst groups within the historical range, and considered chance findings. Thus, the highest dose tested may be considered for developmental effects as the no-observed-adverse-effect level (NOAEL).

5. Conclusions

According to the results of the provided developmental toxicity study on generic pesticide pinoxaden, the NOAEL for maternal toxicity was established at 10 mg/kg/bw/day and LOAEL at 30 mg/kg/bw/day. For developmental toxicity with fetotoxic effects, the NOAEL was determined as $\geq$30 mg/kg/ bw/day, the highest dose tested.

The study demonstrated that the generic formulation of the pesticide pinoxaden is equivalent to the original technical product in terms of developmental effects. However, regarding systemic toxicity, the effects are more pronounced after exposure to the generic formulation. In comparison to the original molecule, which showed no toxic effects at 30 mg/kg bw/day, the generic pinoxaden exhibited maternal toxicity at this dose level.

In addition, the obtained results may supplement the overall safety and toxicity profile of pinoxaden. Nevertheless, the NOAEL determined in this study does not affect regulatory authorities' previously established ADI of 0.1 mg/kg bw/day, which is based on a 2-year toxicity and carcinogenicity study on rats (JMPR, 2005; EFSA, 2013) [8], [9] and supported by a teratology study on rabbits (EFSA, 2013) [9].

CRediT authorship contribution statement

Nadiia Nedopytanska: Formal analysis. **Mojmir Mach:** Writing – review & editing, Supervision, Conceptualization. **Nataliia Bubalo:** Investigation. **Inna Rashkivska:** Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Mykola Prodanchuk:** Resources, Conceptualization. **Yana Kolianchuk:** Writing – review & editing, Methodology, Investigation, Formal analysis, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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