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Developmental toxicity assessment of the new turf herbicide, methiozolin ([5-(2,6-difluorobenzyl)oxymethyl-5-methyl-3,3(3-methylthiophen-2-yl)-1,2-isoxazoline]), in rabbits

Jinsoo Lee [a], Jeongah Song [a], Eun Ju Jeong [a], Moon-Koo Chung [a], Ki-Hwan Hwang [b], Suk-Jin Koo [b], Wook-Joon Yu [a,*]

[a] Division of Toxicological Research and Evaluation, Korea Institute of Toxicology, Daejeon, Republic of Korea
[b] Moghu Research Center Ltd., Daejeon, Republic of Korea

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ABSTRACT

Methiozolin is a new herbicide to control annual bluegrass (*Poa annua* L.) and large crabgrass (*Digitaria sanguinalis* (L.) Scop.) in various turfgrasses. The potential of methiozolin to induce maternal and developmental toxicity was investigated in the pregnant New Zealand White Rabbits. Methiozolin was, at dose levels of 0, 125, 250 and 500 mg/kg/day, administered by oral gavage to artificially inseminated rabbits (25 females per group) from days 6 to 28 of gestation. All does were subjected to Cesarean section on day 29 of gestation. At 500 mg/kg/day, treatment-related toxicities including abortion (10/22), decreased mean body weight, weight gain, net body weight change, reduced food consumption and decreased fetal weight were observed. At 125 and 250 mg/kg/day, no signs of maternal and developmental toxicity were observed. There were no treatment-related external, visceral and skeletal abnormalities of fetuses at all doses tested. In the current experimental conditions, the no observed adverse effect levels (NOAELs) of methiozolin are considered to be 250 mg/kg/day for does and prenatal development.

1. Introduction

The importance of using safe and effective herbicide has increased, because demands of plant resources utilization are rapidly growing due to population growth, climate change and depletion of water supply (Powles and Yu, 2010). Herbicide, as known a weed killer, plays an important role in efficient use of plant resources including a variety of crops and biomass (Pimentel and Pimentel, 1979). Plant resources are so closely related with human life that it is important to use more eco-friendly and non-toxic herbicide to control weed.

Methiozolin is a new turf herbicide in isoxazoline chemistry (Koo et al., 2010, 2014). This new herbicide was initially reported to be useful in controlling barnyardgrass (*Echinochloa* spp.), sedge weeds and several other annual broad-leaved weeds in paddy condition (Hwang et al., 2005). Later, this new molecule was also found to have potent pre- and post-emergence efficacies on annual bluegrass (*Poa annua*) and large crabgrass (*Digitaria sanguinalis*), with high safety to various warm- and cool-season turf grass including Kentucky bluegrass, perennial ryegrass, zoysiagrass, and bermudagrass (Koo et al., 2008). Methiozolin was reported that it mainly inhibited biosynthesis of both cellulose and hemicelluloses fractions (Lee et al., 2007). However, the herbicidal symptom of methiozolin indicated that its mode of action was different from that of inhibitors of cellulose synthesis, microtubule disrupter, or inhibitors of very-long-chain fatty acids (Koo et al., 2008). In addition, it was recently reported that methiozolin might inhibit tyrosine aminotrasferase involved in the plastoquinone biosynthesis in duckweed (*Lemna paucicostata* L.) (Grossmann et al., 2011). In the view of the results so far achieved related with mode of action of methiozolin, the primary mode of action of this new turf herbicide is still unclear (Hwang et al., 2013).

As a part of safety evaluation of methiozolin, acute oral and 90-day repeated dietary administration toxicity studies were already conducted. LD_{50} of acute oral toxicity study was considered to be at least 2000 mg/kg/day, and NOAEL of a 90-day repeated toxicity study were considered to be 5000 ppm in diet (Hwang et al., 2013). These acute and repeated toxicity study results indicate that methiozolin is practically non-toxic to rats. However, the effects of methiozolin on pregnant rabbits and prenatal development have not been investigated. This study was conducted to

* Corresponding author. Fax: +82 42 610 8015.
E-mail address: yuwj@kitox.re.kr (W.-J. Yu).

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evaluate the potential of methiozolin to induce maternal and prenatal toxicity in the New Zealand White Rabbits.

2. Materials and methods

2.1. Animal maintenance and artificial insemination

Approximately 5- to 6-month-old female New Zealand White rabbits were purchased from Orient Bio Inc. (Gyeonggi-Do, Republic of Korea). The animal room was maintained at 17–23 °C and relative humidity of 45–65%. The photoperiod was maintained on a 12-h light cycle (light during 08:00–20:00 with illumination of 150–300 Lux). Standard rabbit diet pellets (Purina Korea Co. Ltd., Seoul, Republic of Korea) and UV-irradiated water were provided *ad libitum*. An artificial insemination technique was used for obtaining pregnant does (Kim et al., 1996). In brief, approximately over 6 month-old twenty-one adult New Zealand White rabbit males were used for semen collection. The semen was collected from bucks and diluted approximately 5-fold with physiological saline. Sperm condition including motility and amount was examined microscopically, and then approximately 0.5 mL of the diluted semen was injected with a glass pipette into the vagina of females. After semen injection, the females were injected with 30 IU of human chorionic gonadotropin (Dae Sung Micro biological Labs Co. Ltd., Kyunggi-Do, Republic of Korea) via a lateral ear vein to confirm ovulation. The day of artificial insemination was designated as day 0 of gestation. Inseminated females were individually housed in stainless steel cages (716 W × 716L × 470H) with a noryl perforated cage bottom and noryl shelf to offer the rabbit a raised area and shelter during the experimental period. In addition, toy was also offered for animal enrichment. Pregnancy was determined with implantation marks on the uterus when caesarean section was performed. This experiment was conducted in facilities approved by the American Association for Accreditation of Laboratory Animal Care (AAALAC) International. All procedures were approved by Korea Institute of Toxicology (KIT) Institutional Animal Care and Use Committee (IACUC).

2.2. Test chemical and treatment

The test item, methiozolin, was supplied by Moghu Research Center Ltd. (Daejeon, Republic of Korea). Methylcellulose was purchased from Sigma–Aldrich Co. Ltd. (St. Louis, USA), and it was dissolved at 0.5% (W/V) in distilled water for injection to prepare the vehicle solution. Methiozolin was suspended at concentrations of 12.5, 25 and 50 mg/mL in 0.5% (W/V) methylcellulose solution. Methiozolin was administered to artificially inseminated New Zealand White rabbits by oral gavage from gestational days 6–28 at a dose volume of 10 mL/kg.

2.3. Experimental groups

Four groups were constructed: Methiozolin 125, 250, 500 mg/kg/day and a vehicle control. Twenty-five inseminated female rabbits were used in each group.

2.4. Dose selection

Doses of 250, 500 and 1000 mg/kg had been given in a dose range-finding study to 6 artificially inseminated females per group. At 1000 mg/kg, only 1 dam survived to scheduled necropsy on gestational day 29. At 500 mg/kg, body weight gain fluctuated during the treatment period from 6 to 29 of gestation. A slightly higher incidence of decreased defecation was observed. At 125 and 250 mg/kg, there were no treatment-related maternal toxicity findings. At Caesarean section, there was no developmental toxicity at all doses tested. Based on these results, 500 mg/kg/day was considered to be for the high dose level. Doses of 250 and 125 mg/kg/day were selected as middle and low doses, respectively, using a common ratio of 2.

2.5. Observation of does

Food consumption, body weights and signs of intoxication were examined in pregnant females. All does were subjected to necropsy on day 29 of gestation. External and internal macroscopic examinations were conducted. The following organ weights were measured at necropsy: heart, liver, spleen, kidneys, adrenal glands and ovaries.

2.6. Caesarean section and fetal examinations

On day 29 of gestation, all pregnant does were subjected to Caesarean section. Gravid uterine was weighed, and then corrected terminal body weight and net body weight change were calculated. All placentas were weighed and examined macroscopically. The number of implantation sites, corpora lutea, live fetuses, dead fetuses, and resorptions (early and late) were counted and recorded. All live fetuses were weighed and evaluated for externally visible abnormalities. They were numbered from the left uterine horn to the right, and then visceral and skeletal examinations were conducted on all fetuses. All live fetuses were examined for visceral abnormality by the Stuckhardt and Poppe (1984) method. Sex of the fetuses was determined at the same time. The brains of fetuses with odd numbers were examined freshly, and those of fetuses with even number were sectioned according to Wilson's method (Wilson, 1965) after fixation with Bouin's solution. All fetuses were fixed with 95% ethyl alcohol and evaluated for skeletal abnormalities after staining according to Dawson's (1926) method. External, visceral and skeletal findings were classified as developmental malformations or variations. We have used the terminology suggested in an internationally developed glossary of terms for structural developmental abnormalities in common laboratory mammals (Makris et al., 2009).

2.7. Statistical analyses of data

The data was analyzed for homogeneity of variance using Bartlett's test. Homogeneous data was analyzed using the Analysis of Variance (ANOVA) and the significance of inter-group differences between the control and treated groups was analyzed using Dunnett's *t* test. Heterogeneous data was analyzed using Kruskal–Wallis test and the significance of inter-group differences between the control and treated groups was assessed using Dunn's Rank Sum test. One-way analysis of covariance (ANCOVA) was used to analyze fetal and placental weight data. The litter size was used as the covariate. Litter data were statistically evaluated using the statistical unit as a litter. The fetal and litter incidence of findings was analyzed using the χ^2-test followed by the Fisher's exact test where necessary. Numerical data obtained during the conduct of the study was subjected to calculation of group means and standard deviations and reported. Data was considered to be significant when $p < 0.05$ or $p < 0.01$. Statistical analyses were performed by comparing the vehicle control group with the treatment groups using Path/Tox System (Version 4.2.2., Xybion Medical Systems Co., USA) or Statistical Analysis Systems (SAS/STAT User's Guide Version 9.2, USA).

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