Hacettepe University
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EFFECT OF THE PENDIMETHALIN ON RAT UTERINE WEIGHT AND GENE EXPRESSION OF mRNAs ENCODING FOR DIFFERENT ESTROGEN-REGULATED GENES ON RAT UTERUS

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Effect of the herbicide pendimethalin on rat uterine weight and gene expression and in silico receptor binding analysis

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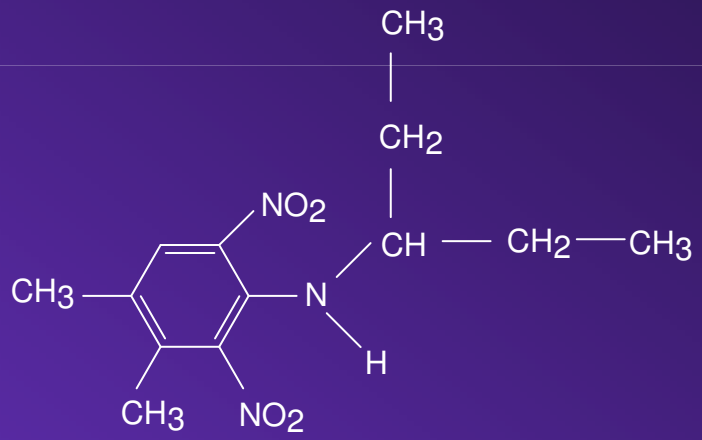
- **Pendimethalin** (N-(1-ethylpropyl)-3,4-dimethyl-2,6-dinitro-benzenamine) is a widely used dinitroaniline herbicide.



- It inhibits the steps in plant cell division responsible for chromosome separation and cell wall formation.

- This pre-emergence herbicide is used to control broadleaf weeds and grassy weed species in cereals, onions, garlic, corn, sorghum, rice, radish, soybeans, peanuts, brassicas, carrots, cabbage, celery, peas, potatoes, cotton, pome fruits, stone fruits, citrus, lettuce, tobacco, and tomatoes.

- In addition, it is used on noncrop areas and on residential lawns, and ornamentals.




- Common usage of pendimethalin in various formulations give rise to its detection as contaminant in soil, ground water, surface water and air.

- After application to soil, pendimethalin may dissipate through evaporation, drift, leaching, and runoff.

- It is degraded through photo-degradation, volatilization or by biodegradation.

- Pendimethalin and its metabolite [4(1-ethylpropyl) amino-2-methyl-3,5-dinitrobenzyl alcohol] are analyzed in a large variety of crops; for most crops, pendimethalin and its metabolite's residue levels were below the limit of quantitation (0.05 ppm).

- Pendimethalin was classified as a ‘slightly toxic’ compound (toxicity class III) and a possible human carcinogen (group C) by the United States Environmental Protection Agency.

Effects on Endocrine System

- Little is known about possible interactions of pendimethalin with endocrine systems.
- According to earlier unpublished studies quoted by regulatory agencies, the thyroid was found to be the most sensitive target in rats.

- Reduced levels of thyroid hormones, increased levels of thyroid-stimulating hormone, thyroid hyperplasia and increased thyroid tumor incidences were reported.

- More recently, pendimethalin has been found to exert agonistic activity at human ER alpha and ER beta, and antagonistic activity at human androgen receptor in vitro in reporter gene assays.

- This prompted us to investigate possible estrogenic actions of this herbicide in an in vivo model system.

- We tested the uterotrophic response to orally applied pendimethalin in the immature rat,
- And effects of this herbicide on the expression of mRNAs encoding for different estrogen-regulated genes,
  - estrogen receptor (ER)-alpha,
  - ER-beta,
  - progesterone receptor (PR),
  - insulin-like growth factor-I (IGF-I),
  - and androgen receptor (AR),

  examined by quantitative real-time RT PCR.

- The study was conducted on Long Evans rats (Møllegaard Breeding and Research Centre, Ejby, Denmark) kept under controlled light and dark cycle (lights on from 02:00 to 16:00 hr) and temperature (22±1^{0}C) with standard diet 3430 and water ad libitum.

- The animal facility was run by the Institute of Laboratory Animal Science, University of Zurich.

- Maximum age of parent animals used for time-pregnant mating was 6 months for females and 12 months for males.
- One receptive female was mated with one male overnight, starting at 16:00h.
- Sperm-positive females were housed in groups of two until 1 day before parturition.

- The day of birth (gestational day 23) was defined as postnatal day (PN) 1.
- On PN 20, the pups were weaned.
- Four to five female littermates were transferred in their home cage to a Techniplast-ventilated storage cabinet for rat cages.

- This cabinet was located in the experimental room, where the animals were again kept under the same light cycle and temperature conditions.

- Animal maintenance and experiments were conducted according to the Swiss Law for the Protection of Animals and the Ethical Guidelines of the Swiss Academy of Medical Sciences.

Treatment schedules

- The acute oral LD_{50} of pendimethalin for female adult rats was given as 1050 mg/kg by US EPA.
- Pendimethalin and ethinylestradiol were dissolved in sterile olive oil containing 2.2 % absolute alcohol.

- In the present study,
  - 150, 225, 300, and 600 mg/kg body weight pendimethalin,
  - 1 µg/kg body weight ethinylestradiol (positive control),
  - or vehicle

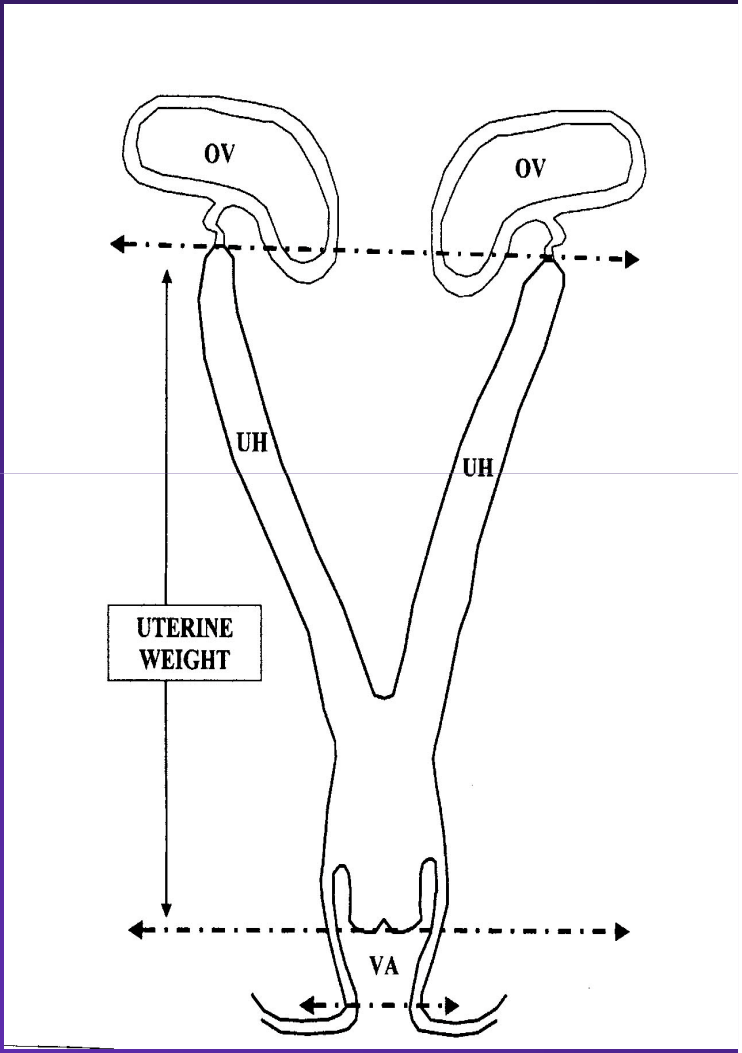
were applied to eight or ten female pups per dose group, once daily by oral gavage, in a volume of 4 ml/kg body weight.

- The chemicals were administered on PN 21, 22 and 23 (corresponding to postnatal days 20, 21, and 22 of the OECD protocol).

Preparation of uterus

- Twenty-four hours after the last gavage, at PN24, the immature females were sacrificed by decapitation under light ether anesthesia.

- The uterus was dissected with cuts between uterine cervix and vagina, and at the top of uterine horns, trimmed free of fat and connective tissue, blotted with sterile gauze to remove the adherent fluid, weighed (wet weight), and frozen in liquid nitrogen until further analysis.



Determination of mRNA by real-time RT PCR

RNA isolation and reverse transcription

- The frozen uterus was thawed and homogenized in RNA lysis (RLT) buffer of the RNeasy-mini kit by a polytron roto-stator homogenizer.

- Total RNA was extracted with RNeasy-mini kit according to manufacturer's instructions. Genomic DNA was thoroughly digested by DNase-I.

- Total RNA concentration was determined by absorption at 260 nm.
- RNA was stored at -80 ^{0}C until use.
- For reverse transcription, 10 µg RNA was used in a total volume of 100 µl containing 1 x TaqMan RT buffer, 5.5 mM $MgCl_2$, 500 µM of each dNTP, 2.5 µM random hexamer primers, 0.4 µM RNase inhibitor, and 1.25 µl MultiScribe™ reverse transcriptase.

- The mixture was incubated for
  - 10 min at 25 ^{0}C,
  - followed by 30 min RT at 48 ^{0}C and
  - 5 min RT inactivation at 95 ^{0}C.
- RT samples were frozen at – 80 ^{0}C.

Primers and TaqMan probes

- Sequences were derived from National Center for Biotechnology Information (NCBI) gene bank.

- Forward and reverse primers and TaqMan probe were designed with PrimerExpress Software, version 2.0 (Applied Biosystems), and ordered from Microsynth (Balgach, Switzerland).

- To exclude amplification from possible DNA contamination, either the probes or the primers were designed to overlap exon junctions in cDNA regions derived from intron-bearing genes.
- All probes were labeled with the fluorescent dyes 6-carboxy-fluorescein (FAM) as reporter and 6-carboxy-tetramethyl-rhodamine (TAMRA) as quencher.

Real-time RT PCR

- Determinations were run on 96-well plates.
- To amplify cDNA, RT samples were mixed with TaqMan PCR master mix (Applied Biosystems), optimized concentrations of primers and probes and distilled water were added to a final volume of 25 µl.
- Signals were monitored by an ABI PRISM 7700 Sequence Detector (Applied Biosystems).

- PCR cycle parameters were used with an initial denaturation step at 95 ^{0}C for 10 min, followed by 40 cycles at 95 ^{0}C for 15 sec and 60 ^{0}C for 1 min.

- Sequence Detector Software SDS 2.0 (Applied Biosystems) was used for data analysis.

- mRNAs were quantified according to the Standard curve method, and normalized to cyclophilin (cyc).

- Cyclophilin was chosen as reference gene, it was found to be comparatively little affected by manipulations of the gonadal axis when compared to other house keeping genes.

Results

Effect of pendimethalin and ethinylestradiol on the immature rat uterus

- During administration of pendimethalin to immature female Long Evans rats, no clinical signs of toxicity or changes in behavior were observed during cage-side observations.
- Uterine weight was measured on PN 24 (day of birth = PN1), 24 hr after the last administration of chemicals.

- The higher two doses of pendimethalin, 300 and 600 mg/kg/day, elicited a small but significant increase in absolute uterine weight.
- Relative uterine weight was increased by 600 mg/kg/day pendimethalin.
- Ethinylestradiol caused a marked increase in uterine weight at 1µg/kg/day (Table 1).
- Body weight was not signficantly changed by pendimethalin or ethinylestradiol.

Table 1. Body weight and absolute and relative uterine weights in immature rats treated with pendimethalin or ethinylestradiol (positive control) for 3 days [a]

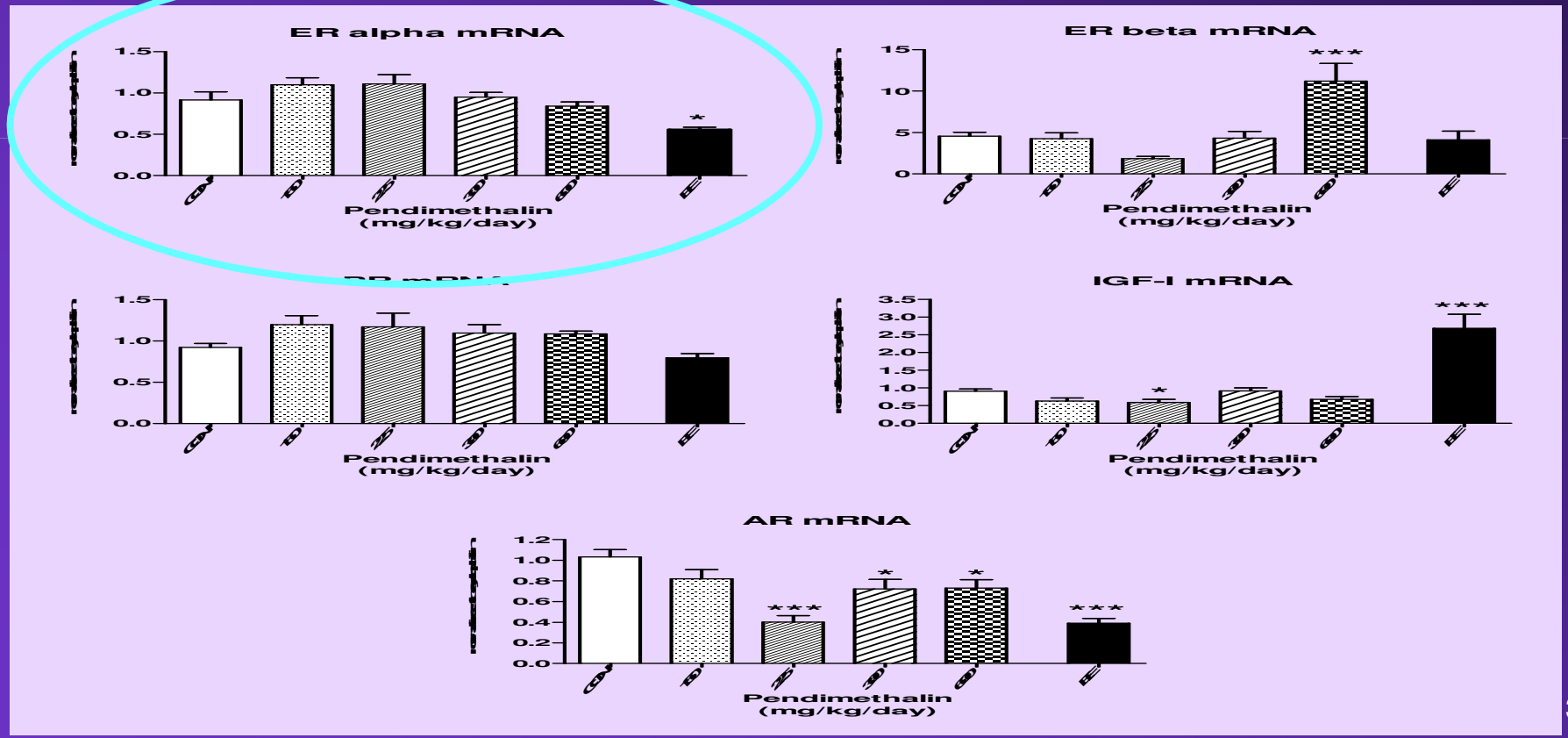
Treatment	Vehicle control	Pendimethalin (mg/kg/day)				Ethinylestradiol 1 µg/kg/day
		150	225	300	600	
Body weight (g)	41.45 ± 5.44 (13)	43.66 ± 4.37 (11)	37.63 ± 3.26 (8)	41.59 ± 4.62 (8)	40.76 ± 3.67 (8)	37.93 ± 1.22 (6)
Absolute uterine weight (mg)	27.55 ± 1.95 (13)	27.78 ± 1.65 (11)	28.98 ± 3.86 (8)	32.38 ± 5.35* (8)	32.06 ± 4.46 * (8)	71.03 ± 9.29 *** (6)
Relative uterine weight (mg/kg bw)	0.674 ± 0.086 (13)	0.642 ± 0.072 (11)	0.776 ± 0.126 (8)	0.781 ± 0.113 (8)	0.786 ± 0.073 * (8)	1.869 ± 0.199 *** (6)

[a] Mean ± SD and number of animals. * p< 0.5, *** p<0.001 different from vehicle-treated group

mRNA levels in uterus of immature rats

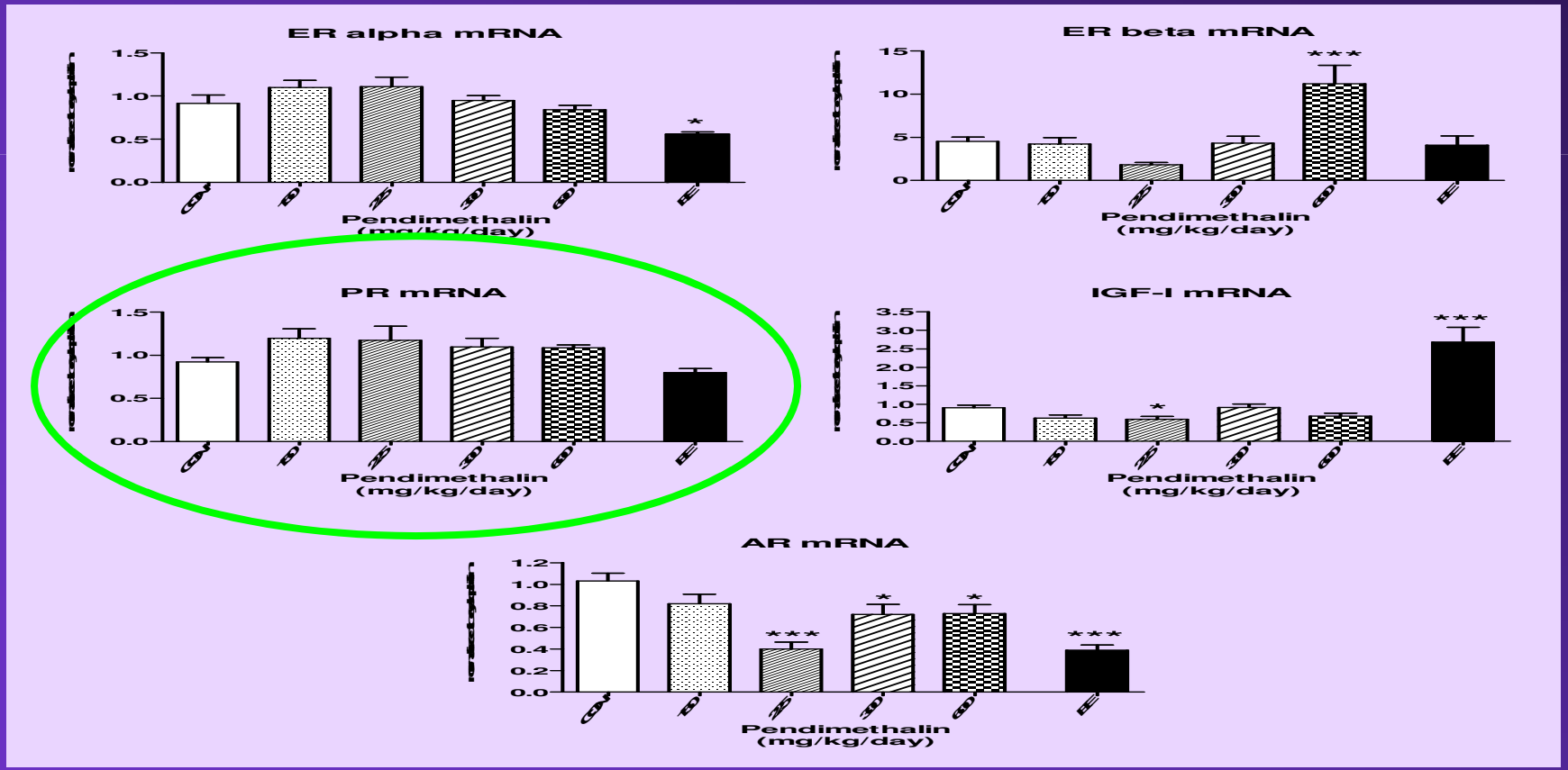
- Expression of the estrogen-regulated genes, ER-alpha, ER-beta, PR, IGF-I, and AR was examined by quantitative real-time RT PCR in the uterus, 24 hr after the last administration of chemicals, with cyclophilin mRNA as reference.

- ER-alpha mRNA expression was not affected by pendimethalin but down-regulated by ethinylestradiol.

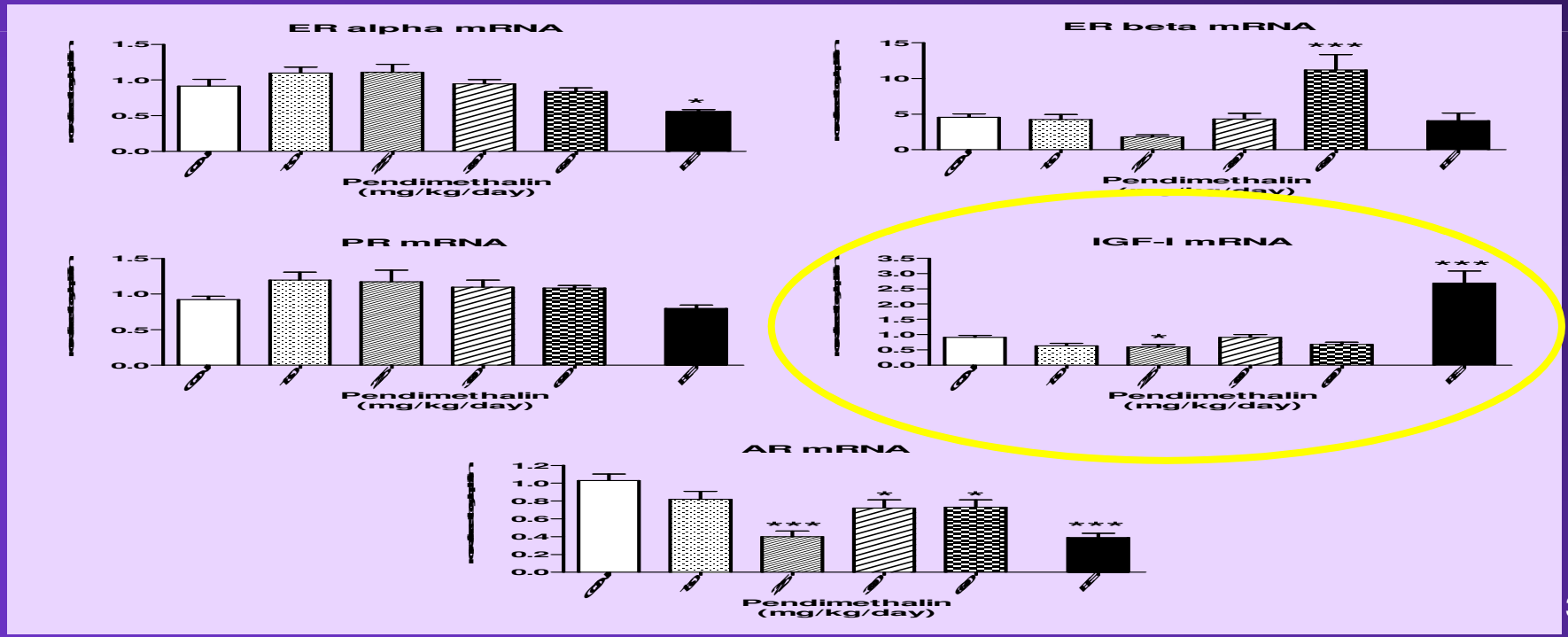


- ER-beta mRNA was significantly increased by 600 mg/kg/day pendimethalin, whereas ethinylestradiol was ineffective at the dose tested.

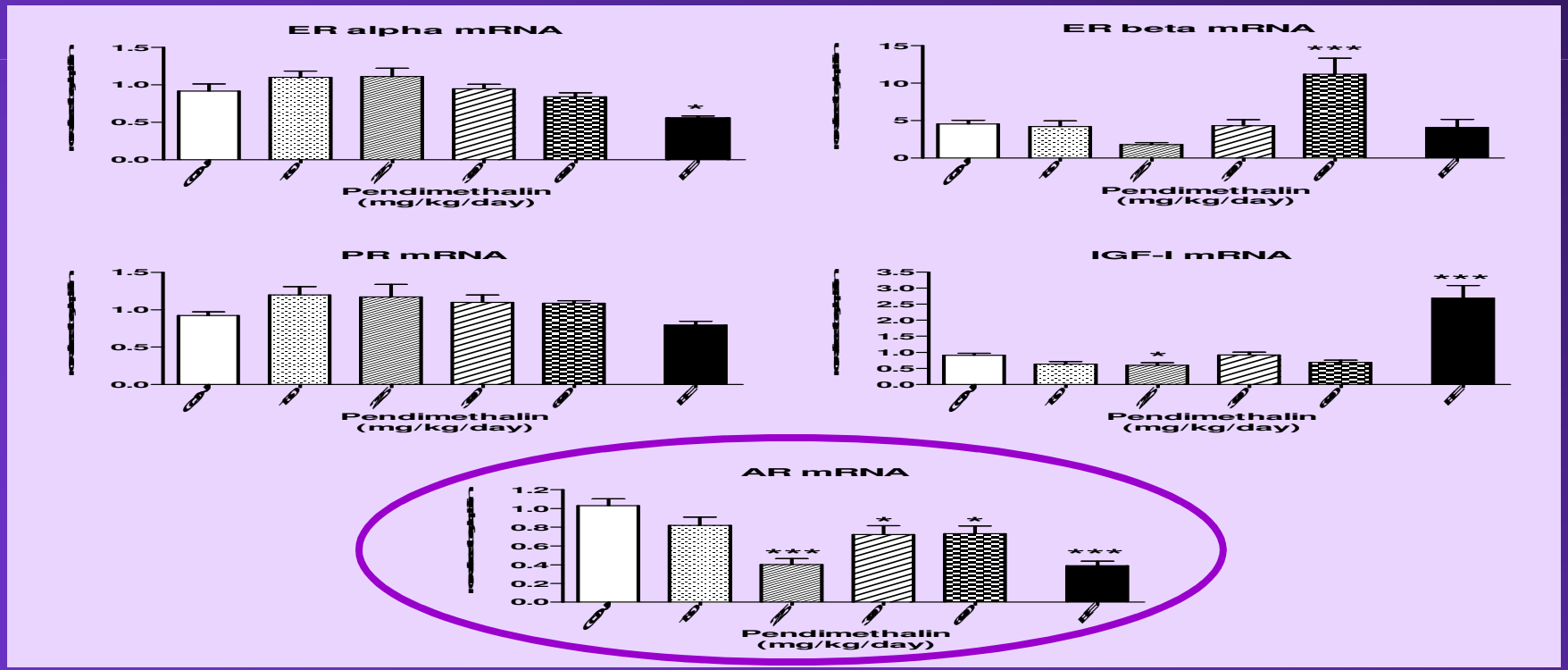
- PR mRNA expression was not significantly changed after repeated administration of either pendimethalin or ethinylestradiol.



- IGF-I mRNA levels showed a decrease after pendimethalin that was significant at 225 mg/kg/day (65.85% of control), and increased after ethinylestradiol.



- A significant decrease of AR mRNA expression was found in rats treated with 225, 300, and 600 mg/kg/day pendimethalin, as well as after ethinylestradiol.



In silico analysis of pendimethalin binding to steroid receptors

- The in silico analysis with VirtualToxLab™ software indicated that pendimethalin could bind to ER-beta and thyroid receptor beta with affinities in the low micromolar range, and to AR with somewhat less affinity.

- In contrast, virtually no binding was found for ER-alpha (Table 2).

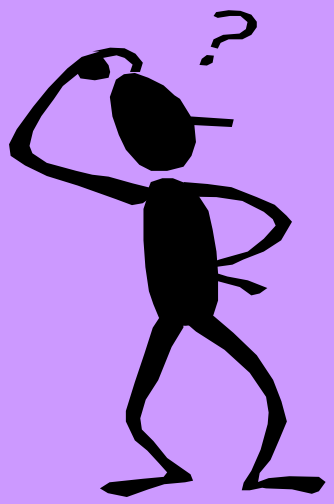
Table 2
In silico prediction of receptor binding characteristics of pendimethalin.

Target protein	Binding affinity from in silico model[a] (IC50)	Stimulation (REC20) or inhibition (RIC20) of transactivation in CHO-K1 cells (Kojima et al., 2004)[b]
Androgen receptor	37.2 ± 5.6 μM	1.2 μM
Aryl hydrocarbon receptor	Not binding	
Estrogen receptor-alpha	Not binding	1.7 μM
Estrogen receptor-beta	3.28 ± 0.37 μM	2.0 μM
Glucocorticoid receptor	0.89 ± 0.17 μM	
Mineralocorticoid receptor	Not binding	
PPAR-gamma	Not binding	
Thyroid receptor-alpha	Not binding	
Thyroid receptor-beta	5.37 ± 1.68 μM	

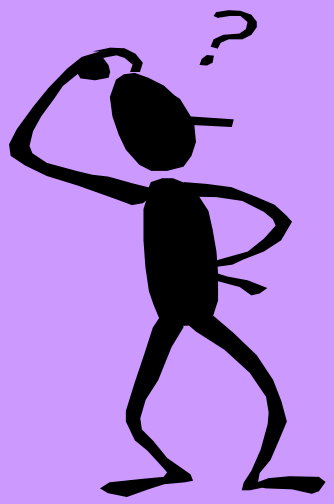
[a] In silico binding affinity calculated with VirtualToxLab™ software. Affinities of ethinylestradiol calculated with the same software: ER-alpha = 8.21 ± 0.42 nM, ER-beta = 3.37 ± 0.18 nM, AR = 1.83 ± 0.23 μM.

[b] Data from Kojima et al. (2004) shown for comparison. Stimulation of transactivation at hER-alpha and h-ER-beta, inhibition of dihydrotestosterone (DHT)-induced transactivation at hAR in Chinese hamster ovary cells. Note that the concentrations yielding 20% of agonist activity of 0.1 nM estradiol (REC20) or 20% inhibition of agonist activity of 0.1 nM DHT (RIC20) are given, rather than ED50 or IC50.

- **In conclusion, the finding of increased absolute and relative uterine weight and altered estrogen target gene expression in uterus of immature rats, indicates that the commonly used herbicide pendimethalin possesses a weak endocrine disrupting potential in vivo.**

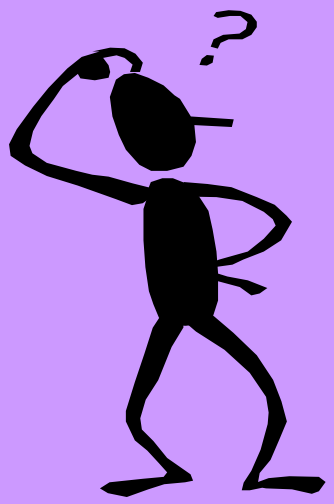


- **The small size of the uterotrophic effect and the expression pattern of several mRNA species (ER-alpha, ER-beta, IGF-I, AR) are compatible with the idea that pendimethalin is an estrogenic substance with predominantly ER-beta activity.**

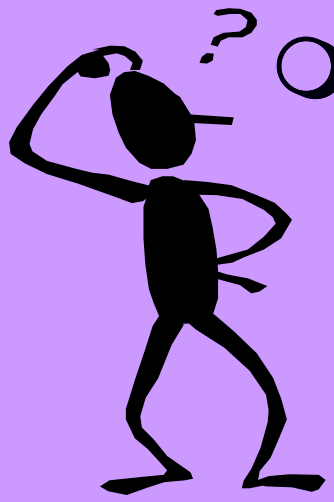


- Estrogen receptor (ER)-alpha mRNA levels were not affected, whereas ER-beta mRNA was up-regulated at the highest dose.

- Progesterone receptor mRNA level was not significantly changed, while insulin-like growth factor-I mRNA was reduced, significantly at 225 mg/kg/day to 65% of control.

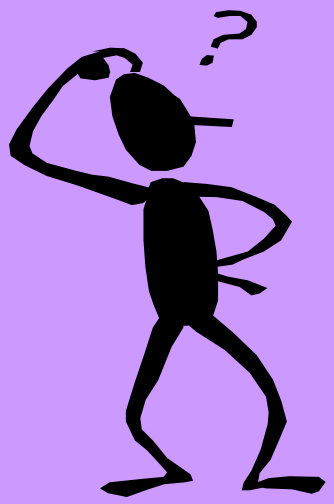


- **Androgen receptor (AR) mRNA showed a marked down-regulation at doses of 225 mg/kg/day and above.**



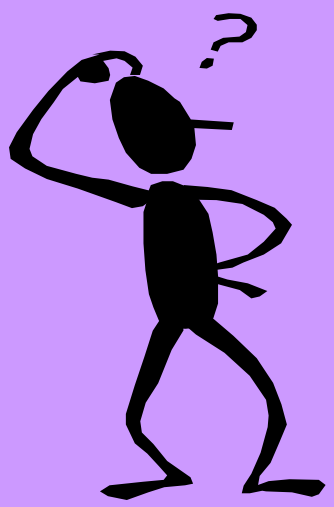
- **The expression pattern differed from that of ethinylestradiol.**

- **In silico analysis revealed potential binding of pendimethalin to ER-beta and AR, but virtually no binding to ER-alpha.**

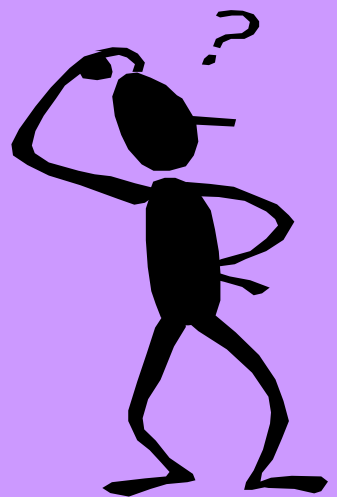


- **These data demonstrate that pendimethalin exhibits estrogenic activity also in vivo.**

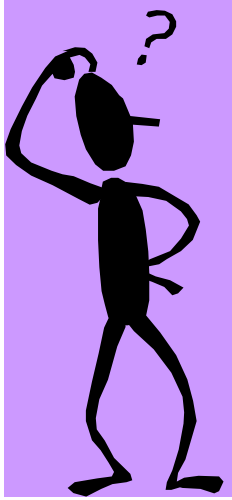
- **However, its uterotrophic effect, which is an ER-alpha-mediated response, is very small, and it appears that in vivo actions should rather be sought in ER-beta-regulated functions.**



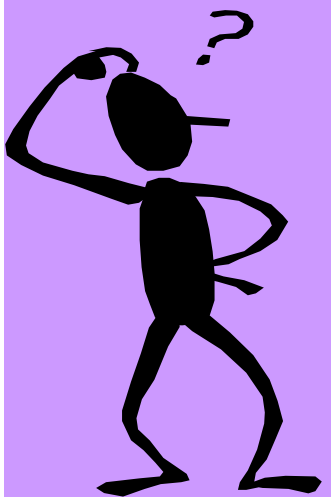
- **The uterotrophic assay does not provide information on chronic exposure conditions.**



- **However, with a uterotrophic NOAEL (no observed adverse effect level) of 225 mg/kg/day, several orders of magnitude above estimated daily exposure levels, e.g., chronic dietary exposure of the general population (0.00041 mg/kg/day) and 1-6 year-old children (0.00087 mg/kg/day), and also occupational exposures, effects on female reproductive organs would hardly be expected from exposure to pendimethalin alone.**



- **But, pendimethalin may contribute to the combined effect of the mixture of endocrine active chemicals present in organisms.**



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