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OFFICE OF
PREVENTION, PESTICIDES AND
TOXIC SUBSTANCES

MEMORANDUM

12/20/99

SUBJECT: Atrazine: Review of a pilot study which evaluated a method of measuring the proestrus afternoon LH and prolactin surges in Sprague-Dawley female rats.

FROM: Roger Hawks, Ph.D.
Reregistration Branch III
Health Effects Division (7509C) *Roger Hawks 12/20/99*

THRU: Jess Rowland, Branch Chief
Reregistration Branch III
Health Effects Division (7509C) *Jess Rowland 1/16/00*

TO: Pam Noyes
Special Review and Reregistration Division (7508C)

DP Barcode: D261334
Submission No.: S571785
Chemical: 080803
Caswell No.: 063

Purpose of Memo - A pilot study (MRID 43934404) was performed in order to determine the validity of a protocol to be used in subsequent studies to measure the proestrus afternoon LH and prolactin surges in the Sprague-Dawley female rat. This study was reviewed and a data evaluation record (DER) of this study was written. The study was found to be **Acceptable-nonguideline**. The study does not satisfy a FIFRA Subdivision F or OPPTS series 870 guideline and was not submitted with the intention of doing so. This study adequately demonstrates the validity of the methods proposed for evaluating LH and prolactin surges.

Primary Reviewer- Roger Hawks, Ph.D., RRBIII
Secondary Reviewer - Melba Morrow, D.V.M., RABI

The DER is attached and the executive summary follows:

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EXECUTIVE SUMMARY:

In a study (MRID 43934404) to determine the validity of a proposed protocol for testing the effect of atrazine exposure on the proestrus afternoon luteinizing hormone (LH) surge, estradiol (E₂) was administered to 70 ovariectomized female Sprague Dawley rats through a surgically implanted silastic capsule. Serum LH, E₂, and prolactin levels were measured 3 days later to determine if the LH and prolactin surges could be measured and to evaluate success of the estradiol implantation procedure.

The ovariectomy followed by estradiol implantation procedure appeared to be effective in inducing LH and prolactin surges, as both these surges were clearly evident in rats undergoing this procedure.

This special study in the rat is **Acceptable-nonguideline**. This study does not satisfy any guideline requirements and was not submitted with the intention of satisfying a guideline requirement.

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[ATRAZINE]

Special Study - Non-Guideline

EPA Reviewer: Roger Hawks, Ph.D.
Reregistration Branch III(7509C)
EPA Secondary Reviewer: Melba Morrow, D.V.M.
RAB I (7509C)

Roger Hawks, Date 12/15/99
M. Morrow, Date 2/17/99

DATA EVALUATION RECORD

DP BARCODE: D261334
P.C. CODE: 080803

SUBMISSION CODE: S571785
TOX. CHEM. NO.: 063

TEST MATERIAL: None

CITATION: Morseth, S. (1996) Evaluation of luteinizing hormone (LH) in female Sprague-Dawley rats- pilot study. Corning Hazelton Inc., Vienna, VA. Laboratory report number: CHV 2386-111. January 18, 1996. MRID:43934404. Unpublished.

SPONSOR: Novartis Corporation (formerly Ciba-Crop Protection) Greensboro, N.C.

EXECUTIVE SUMMARY:

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COMPLIANCE: Signed and dated GLP, Quality Assurance, Data Confidentiality, and Flagging statements were provided.

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I. MATERIALS AND METHODS

A. MATERIALS:

1. Estradiol: β -Estradiol 3-Benzoate
Description: white powder
Lot#: 52H3881
Purity: 98% a.i.
Stability of compound: Stable at room temperature

2. Vehicle and/or positive control: Sesame Oil. Lot no. 113H1212

3. Capsule: The capsule into which the estradiol was inserted was a 20 mm length of silastic tubing which was prepared to an active length of 8 mm.

4. Test animals: Species: Rat
Strain: Sprague-Dawley
Age and weight at study initiation: 9 weeks; Weight at initiation not given
Source: Charles River Labs, Raleigh, N.C.
Housing: During the study the animals were individually housed in stainless steel wire-mesh cages.
Diet: PMI® Feeds, Inc. Certified Rodent Diet #5002. ad libitum
Water: Tap water ad libitum
Environmental conditions:

Temperature: 69 to 78.8°F.
Humidity: 40.5 to 75%
Air changes: ≥ 10 per hour
Photoperiod: 14 hour light/10 hour dark

Acclimation period: Two weeks

B. STUDY DESIGN:

1. In life dates - start: May 24, 1995 end: June 19, 1995

2. Animal assignment - Animals were assigned to the test groups shown in Table 1 using randomly generated numbers from a computer.

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TABLE 1: STUDY DESIGN

Test Group	Clock time ¹	Biologic time ²	Number of females
1	8:00 am	1200	10
2	10:00 am	1400	10
3	12:00 pm	1600	10
4	2:00 pm	1800	10
5	4:00 pm	2000	10
6	6:00 pm	2200	10
7	8:00 pm	2400	10

1 Clock time - This is the actual time when the animals were sacrificed. Because the animals were on their own light cycles which did not necessarily correlate with the time of day as determined by the clock, the biologic time is the more important parameter to examine.

2 Biologic time - The biologic time is the time of day the animals see; the time of day as determined by the animals light cycle. This is the important parameter to consider when estimating when the proestrus afternoon LH surge should be occurring.

3. Rationale and purpose of study - This study was designed to determine the effectiveness of an estradiol capsule implantation procedure and the validity of procedures to measure proestrus afternoon LH and prolactin surges. This study is one of a series of studies (MRIDs 43934404; 43934405; 43934406; and 44152102) which examine the effect of atrazine exposure on, primarily, the proestrus afternoon LH surge. This hormonal surge stimulates ovulation. A delay or lack of ovulation in response to atrazine exposure has been implied in a previous study (MRID 42085001) which examined estrous cycles (through vaginal smears) in SD rats exposed to atrazine. SD rats in this study displayed an increased percentage of days in the estrus phase of the estrous cycle early in the study following atrazine exposure. Increased days in estrus implies a delay or lack of ovulation. A histomorphologic evaluation of the ovaries from this study (MRID 43598622) also showed evidence of a delay or absence of ovulation at the early time points in atrazine-exposed SD females.

The proestrus afternoon LH surge was investigated in this series of studies to examine the possibility that the apparent delay or absence of ovulation seen in MRID 42085001 was due to atrazine's effects on the LH surge.

In addition to the proestrus afternoon LH surge, rats also display a proestrus afternoon prolactin surge. The effects of atrazine exposure on this surge were also investigated in this series of studies.

The effects of atrazine on the LH and prolactin surges are investigated in MRIDs 43934406 and 44152102. The current study (MRID 43934404) and MRID 43934405 - which is reviewed in a separate DER - are, respectively, a pilot study and method validation study in which the methods and protocol used to measure the LH and prolactin surges are tested and validated.

The specific procedures tested in this study were:

- The effectiveness of implantation of silastic estradiol pellets in raising serum estradiol levels to a level sufficient to stimulate an LH surge;
- The utility of using serially sacrificed animals, that is a group of 70 animals with 10 animals sacrificed at 7 time points, to measure LH and prolactin surges (the alternative to serial sacrifice is repeat bleed animals in which the same ten animals would be bled over and over again at each time point);
- The utility of the proposed method of blood collection and hormone measurement (decapitation, plasma fractionation, freezing at -70° , measurement by radioimmunoassay).

The animals were ovariectomized (OVX) in this study. The purpose of OVX followed by implantation of an estradiol-containing pellet was to attempt to "synchronize" the animals. Any given group of normal females will be progressing through the estrous cycle in an unsynchronized fashion. That is, some animals will be in the estrus stage, others in diestrus, and others in proestrus. OVX animals will cease to cycle and are considered to be acyclic. However, because the LH surge is induced by a rapid rise in serum estrogen levels, a young OVX animal is still capable of having an LH surge if it is exposed to high levels of estradiol. OVX followed by implantation of an estradiol-containing pellet was performed in an attempt to synchronize the animals (*i.e.*, to ensure that the LH surges in all the animals were happening at approximately the same time). Measuring LH surges in a group of animals which have not been synchronized is very difficult as each animal will be having an LH surge on a different day of the week. Because an LH surge is only a few hours in duration, one must have some idea of when to look for it in order to find it and quantify it. Thus, the animals were all made acyclic, were implanted at the same time with pellets containing the identical levels of estradiol, and their serum LH levels were all measured at the same time points. This protocol of OVX followed by estradiol implantation, followed by sacrifice has been previously used by other investigators to induce an LH surge in SD female rats (Legan *et al.*, Role of estrogen as initiator of daily LH surges in the ovariectomized rat. *Endocrinology*, 96:50, 1975).

5. Statistics - Group means, standard deviations and standard errors were calculated for each group at each time point for the hormone measurements.

C. METHODS:1. Observations:

Animals were inspected twice daily for signs of toxicity and mortality.

2. Body weight

The study report does not say that body weights were taken, but the study protocol (which is included as Appendix Two to the study) notes that body weights were to be taken once prior to surgery.

3. Food consumption

Neither the study report nor the study protocol note that food consumption was to be determined.

4. Estradiol capsule preparation

Estradiol was added to sesame oil to a concentration of 4 mg/mL and stirred in a beaker heated to 80°C until a solution was formed. This solution was added to a 20 mm in length silastic tubing with an active length of 8 mm. The ends of the tubing were sealed to make the estradiol-containing capsule. The final concentration of estradiol in the capsules was 4 mg/mL.

5. Ovariectomization

The animals were anesthetized under isoflurane inhalation anesthetic and the ovaries were surgically removed through lateral-dorsal incisions. Surgical wounds were closed with wound clips and Vicryl sutures as needed.

6. Estradiol capsule implantation

The estradiol capsules were surgically implanted at the time of ovariectomy.

7. Sacrifice and blood collection

Sacrifice occurred on the third day following OVX and capsule implantation according to the time points given in Table One. Sacrifices were by decapitation without anesthesia. Blood was collected into sodium heparin-containing tubes. The tubes were refrigerated at 4°C until they could be spun down for collection of the plasma fraction.

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8. Hormone measurements

Plasma was collected and shipped on dry ice to Dr. Charles Eldridge of Wake Forest Medical Center in Winston Salem N.C. for evaluation of hormone levels. LH and prolactin were measured by radioimmunoassay. Estradiol was measured using the Coat-A-Count® system provided by DPC Corporation, Los Angeles, CA.

9. Necropsy and Histopathology

Neither macroscopic nor histologic examinations were performed.

II. RESULTS:

1. Estradiol levels

The plasma estradiol levels tended to decrease with time. Estradiol levels are shown in the table below.

Table Two: Plasma estradiol levels by time point. Values given in pg/mL.

1200	1400	1600	1800	2000	2200	2400
= 124.7 SD= 30.4 SE= 9.6	= 110.1 SD= 22.7 SE= 7.2	= 106.9 SD= 15.3 SE= 4.8	= 97.1 SD= 20.7 SE= 5.6	= 76.9 SD= 21 SE= 6.7	= 72.6 SD= 14.8 SE= 4.7	= 72.6 SD= 29.4 SE= 9.3

Data from page 23, current study

2. LH levels

An LH surge was evident upon examination of the LH measurements. The surge appeared to peak at 1600 with a 67% mean increase in LH levels over baseline. Plasma LH levels are shown in the table below. Figure one also displays the LH data.

Table Three: Plasma LH levels by time point. Values given in pg/mL.

1200	1400	1600	1800	2000	2200	2400
\bar{x} = 2408.8 SD= 678.1 SE= 213.8	\bar{x} = 3400.2 SD= 1418.3 SE= 448.8 % ¹ = +41%	\bar{x} = 4018.2 SD= 988 SE= 312.4 %= +67%	\bar{x} = 3683.8 SD= 1755.8 SE= 555.2 %= +53%	\bar{x} = 2688.1 SD= 980.3 SE= 310 %= +12%	\bar{x} = 2500.4 SD= 378.5 SE= 277.8 %= +4%	\bar{x} = 2335.8 SD= 554.5 SE= 175.4 %= -3%

¹ Represents the percentage change from baseline (1200 hours)

Data from page 23, current study

3. Prolactin levels

A prolactin surge was evident upon examination of the prolactin measurement data. The surge peaked at the 1400 biologic time point with a 121% increase over baseline. Plasma prolactin levels are shown in the table below. Figure two also displays the prolactin data.

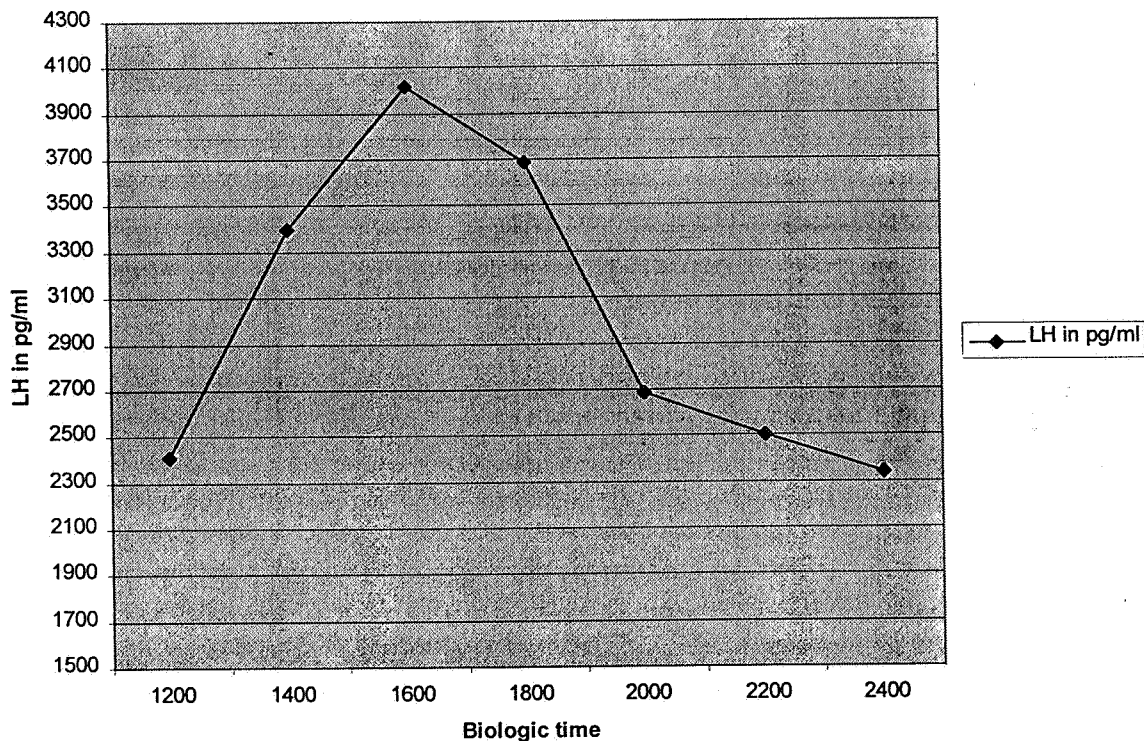
Table Four: Plasma prolactin levels by time point. Values given in ng/mL.

1200	1400	1600	1800	2000	2200	2400
\bar{x} = 18.7	\bar{x} = 41.1	\bar{x} = 32.1	\bar{x} = 27.8	\bar{x} = 22.3	\bar{x} = 20.8	\bar{x} = 10.4
SD= 11.3	SD= 29.3	SD= 12.6	SD= 11	SD= 3.7	SD= 6.8	SD= 5.8
SE= 3.6	SE= 9.3	SE= 3.9	SE= 3.5	SE= 1.2	SE= 2.1	SE= 1.8
	% ¹ = +121%	%= +72%	%= +48%	%= 19%	%= 11%	%= -44%

1 Represents the percentage change from baseline (1200 hours)

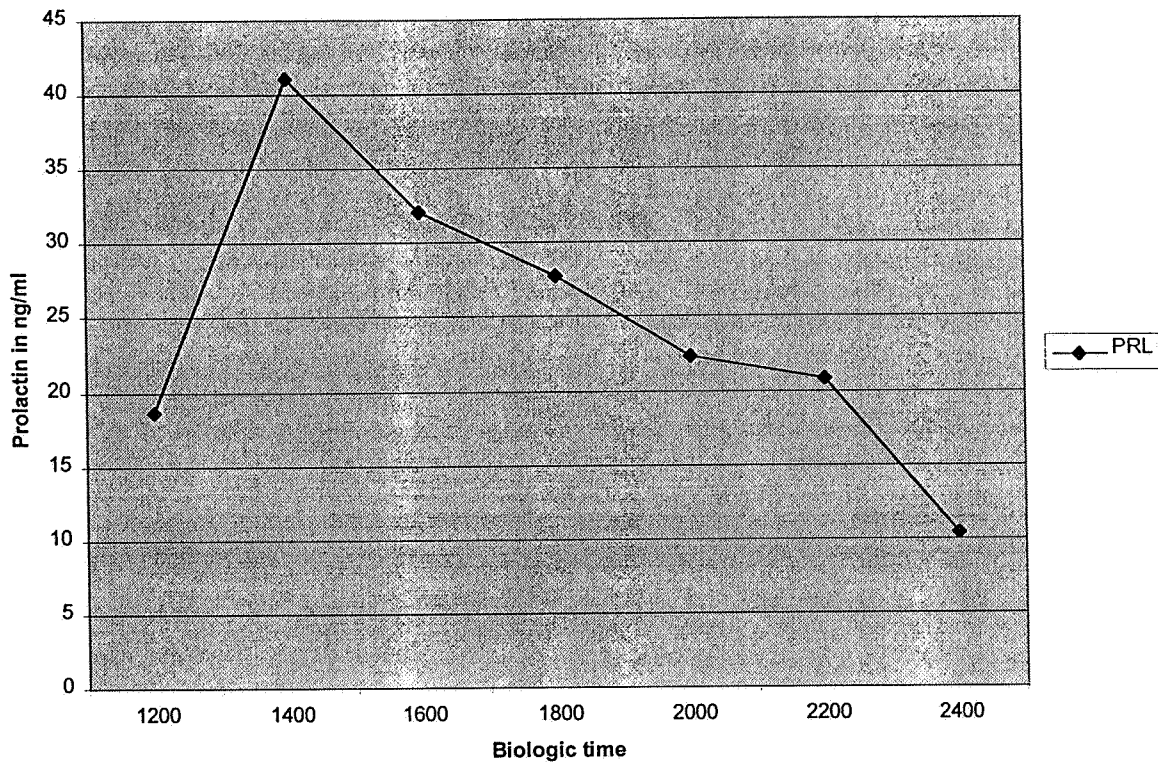
Data from page 23, current study

Figure One: LH Measurements



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Figure Two: Prolactin Measurements



III. DISCUSSION

- A. All animals displayed LH and prolactin surges although the timing of the peaks of the surges varied somewhat. LH values peaked at 1400 hours for 3/10 animals; at 1600 hours for 4/10; and at 1800 for 3/10. Prolactin values peaked at 1400 for 5/10 animals; at 1600 for 4/10 and at 1800 for 1/10. The data shown in Tables 2 and 3 and Figures one and two demonstrate that LH and prolactin surges were occurring in these animals. The procedure of OVX followed by estradiol capsule implantation appears able to stimulate these surges. Using serially sacrificed animals appears to be an appropriate method to use to evaluate these surges. The specific methods used to measure plasma hormone levels (decapitation, plasma fractionation, freezing at -70° , radioimmunoassay), seem to be adequate.

This study is classified as Acceptable-Non-Guideline

The methods used in this study (OVX followed by estradiol capsule implantation, serial sacrifices, and measurement of plasma hormones by radioimmunoassay) appear to be appropriate and valid methods to use to investigate proestrus afternoon LH and prolactin surges in female SD rats.

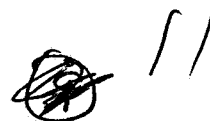
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[ATRAZINE]

Special Study - Non-Guideline

B. Study deficiencies

This study contained minor deficiencies. Body weights do not appear to have been used to randomize the animals. Body weights and clinical signs were not reported, although the protocol on page 30 says these parameters were measured. These deficiencies do not alter the classification of this study as Acceptable-NonGuideline.

A handwritten scribble consisting of several overlapping loops, followed by the number '11' written vertically.