CITATION: Stump DG, 2014. Terbuthylazine: A study of the effects of 4 days of exposure on the estrogen-induced luteinizing hormone (LH) surge in ovariectomized Sprague Dawley rats. WIL Research, 1407 George Road, Ashland, OH 44805-8946, USA. Laboratory Report No. WIL-639222, Audited Draft (26 November 2014). Unpublished. (Syngenta File No. Not yet assigned).

GUIDELINES: This is an investigative study with no applicable guidelines.

GLP: This non-guideline investigative study was conducted in compliance with the United States EPA GLP Standards (40 CFR Parts 160 and 792), 16 October 1989 and 18 September 1989, respectively; the OECD Principles of GLP [C(97) 186/Final], 26 November 1997. The hormone analysis conducted by The University of Arizona Phoenix was not conducted according to GLP standards.

JUSTIFICATION FOR TEST SYSTEM SELECTION: The Sprague Dawley [Crl:CD(SD)] rat was used as the test system on this study. This species and strain of animal is recognized as appropriate for reproduction studies as well as for toxicity studies with atrazine. The number of animals selected was selected to ensure a final minimum number of at least 15 blood samples per group for LH surge assessment. This was the minimum number of samples required for meaningful analysis of the hormone and plasma analysis data.

Four previous studies conducted at WIL Research (Sawhney Coder 2010 and 2011) have demonstrated that atrazine administered by oral gavage at doses of 50 or 100 mg/kg/day for 4 days suppressed the estrogen-induced LH surge in intact as well as ovariectomized, estrogen-induced female Sprague Dawley rats (approximately 13 week old). Foradori *et. al.* (2009) reported that 4 days of atrazine administration in approximately 13-week old female Wistar rats also suppressed the estrogen-progesterone induced LH surge. As the purpose of the current study was to assess the effects of terbuthylazine administered for 4 days on the estrogen-induced luteinizing hormone (LH) surge in ovariectomized young-adult Sprague Dawley rats, atrazine was used as the positive control substance for comparison.

The selected route of administration for this study was oral (gavage) because this is a potential route of exposure for humans. Historically, this route has been used extensively for studies of this nature.

EXECUTIVE SUMMARY

In a study to assess the effects on the hormone-induced luteinizing hormone (LH) surge in ovariectomized female rats, three groups of 23, 20 and 21 ovariectomized female Sprague Dawley rats were dosed by oral gavage with 1% carboxymethylcellulose in deionized water (vehicle control group), 100 mg atrazine/kg/day (positive control group) or 106.5 mg terbuthylazine /kg/day (molar equivalent of 100 mg/kg atrazine), respectively. The animals were ovariectomized and a subcutaneous silastic capsule containing 4 mg/mL of estradiol benzoate in sesame oil was implanted to induce afternoon light-entrained, LH surges. On the fourth day following ovariectomy, plasma samples were taken (over the course of 12 hours) and analysed for LH levels using radioimmunoassay.

Treatment of ovariectomized female Sprague Dawley rats with terbuthylazine resulted in a statistically significant reduction in the estradiol-induced LH surge with a significant main effect of treatment, time, and an interaction effect of time and treatment. The peak LH level of the terbuthylazine treated group was reduced to approximately 47% of the control levels while the peak LH level of the atrazine treated group was reduced to approximately 69% of the control level. Both terbuthylazine and atrazine significantly decreased the area under the curve (AUC) of the LH surge.

Adverse effects, including increased mean body weight losses, lower mean food consumption, and lower mean body weights, were noted in the 106.5 mg/kg/day terbuthylazine group. Similar effects on body weight loss, food consumption, and body weight were noted for females in the 100 mg/kg/day atrazine group.

Conclusion: Terbuthylazine reduced the magnitude of the LH surge in female SD rats when administered for 4 days by oral gavage at a dose of 106.5 mg/kg/day. This effect was comparable to that observed in SD rats administered atrazine for 4 days at the molar equivalent dose of 100 mg/kg/day.

MATERIALS AND METHODS

Materials:

Test Material:	Terbuthylazine
Description:	Technical, white powder
Source:	Syngenta Crop Protection Inc., St. Gabriel, LA, USA
Lot/Batch number:	Batch ID Number 544309, Lot GBL6K26
Purity:	97.4 % a.i (w/w)
CAS#:	Not reported
Stability of test compound:	Recertification date end May 2015 (stored at <30°C)

Positive Control Material:	Atrazine
Description:	Technical, white powder
Source:	Syngenta Crop Protection LLC, St. Gabriel, LA, USA
Lot/Batch number:	Batch Number 726718, Lot GBL0E05 (US)
Purity:	97.5 % a.i w/w
CAS#:	Not reported
Stability of test compound:	Recertification date end May 2017 (stored at <30°C)

Vehicle and/or positive control: 1% carboxymethylcellulose (CMC) in deionized water.

Test Animals:

Species	Rat
Strain	Sprague Dawley [Crl:CD(SD)]
Age/weight at dosing	Approx. 12 weeks of age (sexually mature females) / 232-283 g
Source	Charles River Laboratories Inc., Raleigh, NC, USA.
Housing	Individually in stainless steel wire-mesh cages suspended above cage board
Acclimatisation period	Minimum 9 days including acclimation to intubation i.e. daily dosing of the vehicle at 5mL/kg bw beginning 7 days prior to the first dose administration
Diet	PMI Nutrition International, LLC Certified Rodent LabDiet® 5002, ad libitum
Water	Reversed-osmosis purified drinking water *ad libitum*
Environmental conditions	Temperature: 21.3-21.8°C Humidity: 45.7-77.7% Air changes: 10/hour Photoperiod: 14 hours light, 10 hours dark

Study Design and Methods:

In-life dates: Start: 26 June 2014 End: 01 July 2014

Animal assignment: Animals were randomly assigned to three groups based on stratification of body weight in a block design.

Table 1: Study design

Test group	Dose (mg/kg bw/day)	Number of female rats
Vehicle control	0	23
Atrazine (positive control)	100	20
Terbuthylazine	106.5	21

Dose formulation and analysis: Terbuthylazine, 21.3 mg/mL, and atrazine, 20 mg/mL, were formulated in 1% carboxymethylcellulose (CMC) in deionized water for dosing. Test substances were formulated the day prior to use and stirred continuously overnight and throughout the preparation, sampling and dose administration procedures. One of the terbuthylazine and one of the atrazine dose formulations were analyzed for test substance homogeneity by WIL Research Laboratories using a validated HPLC method and UV absorbance detection.

Resuspension homogeneity and stability of terbuthylazine suspensions (0.1 and 125 mg/mL) in aqueous carboxymethylcellulose (medium viscosity; 1% w/v) following room temperature storage for a minimum of 24 hours has been previously established (WIL-639216). Resuspension homogeneity and stability of atrazine suspensions (0.1 and 50 mg/mL) in aqueous carboxymethylcellulose (medium viscosity; 1% w/v) following room temperature storage for 24 hours was established in a concurrent study (WIL-639223).

Analysis results: Dose formulations for terbuthylazine and atrazine were homogenous and analysed concentrations were within the range of 85% to 115% (98.1% and 101% of nominal concentration, respectively).

Preparation of Estradiol Implants: A 4-mg/mL stock solution of estradiol benzoate in sesame oil was prepared and encased in silastic tubing.

Ovariectomy and Implantation of Catheters and Estradiol Implants: Surgeries were performed (06.00-12.00 hours) on all females 3 days prior to the scheduled blood collection for LH analysis (study day 0). On the day of scheduled surgery, each female was anesthetized with isoflurane. A catheter was implanted in the femoral vein and tunnelled subcutaneously to exit in the scapular region, to allow rapid collection of multiple blood samples. Bilateral ovariectomy was then performed. An estradiol implant placed subcutaneously in the right flank region was used to maintain blood estradiol at levels comparable to those seen in intact females and to produce a daily afternoon surge of luteinizing hormone. This provided a synchronized cohort of animals in which the timing and amplitude of each hormone surge could be evaluated.

Treatment: The vehicle, test substance, and positive control substance formulations were administered orally by gavage once daily for 4 consecutive days (study days 0-3). Dosing began immediately following lights-on (05.00 hours) each day. A dose volume of 5 mL/kg was used.

Investigations: All rats were examined and weighed daily. Food consumption was measured daily. On the morning following the last blood sample collection, females were euthanized by carbon dioxide inhalation. All females (including those found dead or euthanized *in extremis*) were examined for the absence of ovaries, the presence of the estradiol capsule, and the presence and placement of the femoral vein catheter.

Blood samples for LH analysis: Blood samples of approximately 0.5 mL/animal/time point were collected 3 days following ovariectomy following the fourth day of treatment (study day 3) at approximately 11.00, 14.00, 16.00, 18.00, 20.00 and 23.00 hours. During the dark photoperiod (19.00 – 05.00 hours) blood samples were collected under red light. The samples were collected into chilled tubes containing sodium heparin as the anticoagulant. Plasma was isolated in a refrigerated centrifuge and stored at approximately -70°C until analysis at the University of Arizona Phoenix, Phoenix, AZ, USA.

Radioimmunoassay for luteinizing hormone: Plasma LH levels were measured using reagents provided by the National Hormone and Peptide Program (Torrance, CA, USA). Rat LH-RP3 was used to create a standard curve (range:102.5 - 0.0049 ng). Tracer LH was iodinated using the chloramine-T method by the Colorado State University peptide assay core. Samples (10-25 µL per animal/time) were incubated overnight at 4°C with antiserum (NIDDK-Anti-rLH-SIII; diluted 1:300,000). Following incubation, iodinated ovine LH (oLH, NHPP) was added to each tube (~10,000 cpm/tube)

and incubated overnight at 4°C. Bound LH was separated from free LH by incubation with goat anti-rabbit γ-globulin (Calbiochem, cat#539845; 1:1000) in a 5% polyethylene glycol (Fisher Biotech; BP233-1) solution. The intra-assay variance, as measured by internal quality controls, was 5.4%.

Statistics: Analyses were conducted using two-tailed tests (except as noted otherwise) for minimum significance levels of 1% and 5%, comparing the test substance-treated group and the positive control substance-treated group to the control group. Mean body weight, body weight change, and food consumption data were subjected to a two-sample t-test (Sokal and Rohlf, 1981).
All LH data were analysed by a two-way ANOVA to determine treatment, dose group, and interaction effects and a two-way ANOVA with repeated measures across time. Because there were several animals with incomplete LH profiles, these animals were excluded from the analysis in the two-way ANOVA using repeated measures. Comparisons of LH levels at specific time points were done using the Bonferroni correction for multiple comparisons. The peak amplitude of the LH surge was calculated by determining the highest concentration of LH achieved during the sampling period, regardless of timing. The LH levels were plotted using all time points, and the area under the curve (AUC) for each hormone was determined using Prism 5 software (GraphPad Software, San Diego, CA, USA) with baseline set at 0. Peak amplitude and AUC were analysed by one-way ANOVA. The level of statistical significance was set at $p \leq 0.05$ for all statistical tests.

RESULTS AND DISCUSSION

Effects on the luteinizing hormone surge: Terbuthylazine at the same molar equivalent dose as atrazine (positive control) showed a significant reduction in the estrogen-induced LH surge. Two-way ANOVA (treatment x time) analysis of plasma LH using all data points showed significant main effects of treatment [$F_{2,308} = 9.976$, $p < 0.0001$], and time [$F_{5,308} = 22.71$, $p < 0.0001$], and an interaction effect [$F_{10,308} = 3.649$, $p = 0.0001$]. A significant decrease in LH was observed for atrazine and terbuthylazine compared to the vehicle control at the 16.00 h time point based upon a Bonferroni post-hoc analysis. There was a significant decrease at the 18.00 h sampling time point for both atrazine and terbuthylazine compared to the vehicle control.

Table 2: Summary of plasma luteinizing hormone (LH) levels (ng/mL)

Treatment group		Time of day					
		11:00	14:00	16:00	18:00	20:00	23:00
Vehicle	Mean	0.15	0.40	1.71	1.31	0.81	0.18
	SEM	0.01	0.05	0.28	0.23	0.08	0.01
	N	20	20	20	20	20	17
100 mg/kg bw atrazine	Mean	0.14	0.22	0.72***	0.74*	1.03	0.17
	SEM	0.01	0.03	0.23	0.18	0.26	0.01
	N	19	19	18	18	18	14
106.5 mg/kg bw terbuthylazine	Mean	0.14	0.16	0.56***	0.64**	0.77	0.16
	SEM	0.004	0.02	0.09	0.11	0.13	0.01
	N	17	17	18	18	17	15

Bonferroni Post-hoc Analysis - statistically significance * ($p<0.05$), ** ($p < 0.01$), *** ($p<0.001$)

Peak LH level of the terbuthylazine treated group was approximately 47% of the vehicle control group and the atrazine-positive control group peak LH was approximately 69% of vehicle control group. There was a significant reduction in peak LH amplitude between treatment groups compared to vehicle controls group [$F_{2,54} = 5.47$, $p = 0.0192$]. Post hoc analysis using Bonferroni's multiple comparison tests showed significant reductions ($p < 0.05$) in the terbuthylazine group compared to vehicle control but no significant difference between the vehicle control and atrazine groups.

Terbuthylazine as well as atrazine significantly reduced the LH AUC compared to the vehicle group [$F_{2,46} = 97.40$, $p = 0.0023$]. Post-hoc analysis with Bonferroni's multiple comparison tests showed significant reductions in LH AUC in the atrazine ($p<0.05$) and terbuthylazine ($p<0.01$) groups versus vehicle controls with no difference between the terbuthylazine and atrazine groups.

Table 3: Summary of plasma luteinizing hormone (LH) level (ng/mL): LH peak and LH AUC

Treatment group	Peak LH amplitude		LH area under the curve	
Vehicle	Mean	2.04	Mean	9.36
	SEM	0.29	SEM	0.99
	N	20	N	20
100 mg/kg bw atrazine	Mean	1.41	Mean	6.24*
	SEM	0.32	SEM	1.04
	N	18	N	18
106.5 mg/kg bw terbuthylazine	Mean	0.96*	Mean	4.99**
	SEM	0.11	SEM	0.61
	N	17	N	17

One-way ANOVA - statistical significance* ($p<0.05$), ** ($p<0.01$)

Mortality and clinical observations: No terbuthylazine-related deaths, moribundity, or clinical observations were noted (one female was found dead and 2 females were euthanized *in extremis*). Clinical findings of pale and cool extremities, a cool and pale body, red material around the nose, and yellow material around the urogenital area were observed. The death, moribundity and clinical findings were attributed to the surgical procedure.

No atrazine-related death or moribundity was noted. Two females were found dead due to the surgical procedure. Clinical findings were noted infrequently and/or at similar frequencies to the control group.

Body weight and food consumption: Terbuthylazine or atrazine-related greater mean body weight losses, with corresponding lower mean food consumption, were noted for females in the 106.5 mg/kg/day terbuthylazine and 100 mg/kg/day atrazine groups, when compared to the control group during the first 2-3 days of treatment. As a result, lower mean body weights (up to 7.2% and 4.9%, respectively) were recorded.

Table 4: Summary of body weight changes and food consumption

Treatment group	Mean body weight change (g): days 0-4		Mean food consumption (g/rat/day): days 0-4	
Vehicle	Mean	-11	Mean	16
	SD	6.1	SD	1.6
	N	20	N	20
100 mg/kg bw atrazine	Mean	-23**	Mean	13
	SD	9.4	SD	2.5**
	N	18	N	18
106.5 mg/kg bw terbuthylazine	Mean	-28**	Mean	10**
	SD	11.3	SD	3.6
	N	17	N	17

* statistical significance * ($p<0.05$), ** ($p<0.01$),

Necropsy: For all females, the ovaries were absent, the estradiol capsule was present, and the femoral vein catheter was in place at the time of euthanasia.

CONCLUSION: Terbuthylazine reduced the magnitude of the LH surge in female SD rats when administered for 4 days by oral gavage at a dose of 106.5 mg/kg/day. This effect was comparable to that observed in SD rats administered atrazine for 4 days at the molar equivalent dose of 100 mg/kg/day.

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