Human Reproduction Vol.20, No.11 pp. 3018–3025, 2005 doi:10.1093/humrep/dei194
Advance Access publication August 5, 2005.

Effects of the insecticide amitraz, an α_2-adrenergic receptor agonist, on human luteinized granulosa cells

Fiona M.Young[1,5], Margaret F.Menadue[2] and Tina C.Lavranos[3,4]

[1]Department of Medical Biotechnology, School of Medicine, Flinders University, Adelaide, SA 5042, [2]Endocrinology Unit, Flinders Medical Centre, Adelaide, SA 5042 and [3]Department of Obstetrics and Gynaecology, School of Medicine, Flinders University, Adelaide, SA 5042, Australia
[4]Present address: Bionomics Ltd, 31 Dalgleish St, Thebarton, Adelaide, SA 5031, Australia

[5]To whom correspondence should be addressed. E-mail: fiona.young@flinders.edu.au

BACKGROUND: Amitraz, an insecticide used to prevent tick and mite infestation of cattle, crops and dogs, is an α_2-adrenergic receptor agonist that inhibits GnRH release and the ovulatory LH surge in rats. Noradrenalin, the physiological ligand for adrenergic receptors, inhibits progesterone production by IVF-derived granulosa cells, but the effects of amitraz are unknown. METHODS: Luteinized granulosa cells obtained from women undergoing ovarian stimulation were exposed to amitraz (1, 10, 50, 100 μg/ml) for 2–72 h, and to amitraz (50 μg/ml) ± hCG or the specific α_2-adrenergic receptor antagonist yohimbine, for 6 h. Cell numbers were determined by 3-(4, 5-dimethylthiazol-$_2$-yl)-2, 5-diphenyl tetrazolium bromide(MTT) assay and hormone production by radioimmunoassay. RESULTS: Amitraz 10 μg/ml did not affect cell numbers or estrogen production, but reduced progesterone production to 58 ± 8% ($p < 0.01$, 24 h, $n = 6$) of control values. Amitraz (100 μg/ml) was cytotoxic and caused a corresponding reduction in hormone production. Amitraz 50 μg/ml did not affect cell numbers or estrogen production, but reduced progesterone per cell production to 82 ± 6% of control values after 6 h. This was prevented by 0.2 mmol/l yohimbine. Exposure to amitraz 50 μg/ml for 6 h exposure abolished hCG-stimulated progesterone production but not estrogen production. CONCLUSIONS: Amitraz inhibited basal and hCG-stimulated progesterone but not estrogen production. The inhibitory action of amitraz and its antagonism by yohimbine suggest that α_2- adrenergic receptors are expressed by luteinized human granulosa cells.

Key words: α_2-adrenergic receptor/amitraz/human luteinized granulosa cells/reproductive toxicology/steroid hormones

Introduction

Amitraz is an insecticide used to prevent tick and mite infestation (Hollingworth, 1976) and is in common use around the world. Amitraz is applied to cattle (McDougall and Lewis, 1984) and sheep in dip baths at concentrations of 0.025% (Eamens *et al.*, 2001; Mekonnen, 2001), to dogs from collars impregnated with 0.025% amitraz, or by topical application in a bath of 0.05% amitraz (Paradis, 1999; Shaw and Foster, 2000; Elfassy *et al.*, 2001), to pigs in sprays containing 12.5%, and to cotton and hops (Weichel and Nauen, 2003) by spraying 20% solutions of amitraz from aeroplanes and ground sprinklers. In addition, amitraz is used to control psylla infestations of pears (Gosselin *et al.*, 1984; Schaub *et al.*, 2002). Human exposure to amitraz occurs when diluting the concentrate obtained from the manufacturer, when applying the amitraz to crops or animals, and when working in amitraz-treated areas, for example pear orchards or cotton fields (US Environmental Protection Agency, 1996). To our knowledge, there are no controlled studies describing amitraz exposure in humans.

In women, follicles within the ovaries consist of an oocyte surrounded by granulosa and theca cells, which together secrete estrogen until the mid-cycle surge of LH causes the oocyte to be ovulated and the follicular cells to differentiate into the granulosa-lutein and theca-lutein cells of the corpus luteum, which secrete both estrogen and progesterone. Agents that either change the ratio of estrogen : progesterone, or which prevent the production of steroid hormones by luteinized granulosa cells *in vitro*, have the potential to be reproductively toxic to women *in vivo*.

Amitraz is an α_2-adrenergic receptor (α_2-AR) agonist that adversely affects the mammalian reproductive system by binding to presynaptic α_2-AR in the hypothalamus, thus inhibiting noradrenalin release and decreasing GnRH secretion (Altobelli *et al.*, 2001).

When 30 mg/kg of amitraz was administered to rats, the ovulatory LH surge was prevented (Goldman and Cooper, 1993). Male mice that ingested 80 p.p.m. (~0.008%) amitraz for 12 weeks prior to mating had significantly reduced sperm production and sired fewer pups than controls (Al-Thani *et al.*, 2003). The reproductive lowest observed effect level for amitraz was derived by exposing three generations of rats to 50 p.p.m. (~0.005%) amitraz. This exposure resulted in reduced litter size and decreased

pup survival in all three generations. Amitraz exposure of 200 p.p.m. (~0.02%) resulted in insufficient pups to continue F_2 matings (US Environmental Protection Agency, 1996).

Amitraz inhibited insulin but stimulated glucagon secretion in a perfused rat pancreas model (Abu-Basha *et al.*, 1999). Amitraz also decreased intestinal motility (Pass and Mogg, 1991), and inhibited prostaglandin synthesis by bovine seminal vesicle microsomes (Yim *et al.*, 1978). Total immersion of cats in a solution of 0.025% amitraz resulted in reduced food intake and disrupted excretion, and the cats 'appeared sedated and sat quiet and immobile in their cages' (Gunaratnam *et al.*, 1983); however, they had recovered completely within 3 days. The topical application of amitraz to dogs suppressed insulin release and increased plasma glucose (Hsu and Schaffer, 1988). The topical application of amitraz (12.5%) to dermal scabies resulted in 14 children being admitted to hospital with bradycardia or tachycardia, myosis and hyperglycaemia. These symptoms were apparent 9.2 ± 5.5 min after dermal exposure (Kalyoncu *et al.*, 2002), suggesting that amitraz can be absorbed through the skin and can exert systemic effects via the vascular system in humans. The US Environmental Protection Agency classified amitraz as a Category II dermal toxin to humans; this is the second highest of four categories (US Environmental Protection Agency, 1996).

The dose-dependent inhibition of noradrenalin release by amitraz in rat hypothalamic synaptosomes (Altobelli *et al.*, 2001) and inhibition of insulin release by a rat β-cell line (Chen and Hsu, 1994) were reversed by yohimbine, a potent α_2-AR antagonist (Lalchandani *et al.*, 2002). Yohimbine bound to α_2-ARs with pKi values of 8–9.7, but did not antagonize α_1 or β-ARs (Lalchandani *et al.*, 2002).

Noradrenalin is the physiological ligand for both α- and β-ARs (Fohr *et al*, 1993), and inhibited basal (Bodis, *et al*.,1993) and hCG-stimulated progesterone production (Fohr *et al*., 1993) by human IVF-derived luteinized granulosa cells *in vitro*. The β-AR antagonist propranolol reduced the noradrenalin-mediated inhibition of basal progesterone production (Bodis, 1993) but had no effect on hCG-stimulated progesterone production (Fohr *et al*., 1993). The specific α-AR agonist phenylephrine, like noradrenalin, inhibited hCG-stimulated progesterone production, suggesting that the noradrenalin-mediated reduction of hCG-stimulated progesterone production occurred via the α-AR.

Amitraz, like noradrenalin, is an α-AR agonist, and noradrenalin reduced hCG-stimulated progesterone production by human IVF-derived luteinized granulosa cells (Fohr *et al*., 1993). There are currently no data regarding the effects of amitraz on any human cell type; therefore, we aimed to determine whether amitraz has the same effects as noradrenalin on human IVF-derived luteinized granulosa cells; that is, whether amitraz will reduce hCG-stimulated progesterone production and whether the inhibitory action of amitraz will be prevented by the α_2-AR antagonist yohimbine.

Materials and methods

Granulosa cell collection

Human granulosa cells were obtained from informed patients who consented to donate their granulosa cells in a protocol approved by the Flinders Clinical Research Ethics Committee (47/00). The patients were undergoing IVF or ICSI procedures at Reproductive Medicine, Flinders Medical Centre, South Australia. Multiple follicular development was induced after GnRH down-regulation with Synarel (Serono Laboratories, Sydney, Australia), followed by stimulation with recombinant human FSH (Gonal F75; Serono Laboratories). HCG (5000 IU; Profasi; Serono Laboratories) was administered 34–36 h before oocyte retrieval. The oocytes were removed from the follicular fluid and processed for fertilization.

The follicular aspirates from each patient were pooled, centrifuged (1500 *g*), for 5 minutes resuspended in culture medium, Ham's F12/Dulbecco's Minimum Essential Medium (F12/DMEM, 1 : 1; Thermo Trace, Melbourne, Australia.) supplemented with 10% fetal calf serum (Trace Biosciences) and insulin, transferrin, selenium (ITS; Invitrogen Life Technologies, Mount Waverley, Australia). Erythrocytes were removed by centrifugation through Lymphoprep (1 g/ml; Axis Shield, Oslo, Norway). Collected granulosa cells were washed and counted using trypan blue exclusion, then distributed into 96-well plates in 100 μl of supplemented F12/DMEM and incubated in a humidified atmosphere of 5% CO_2 in air at 37°C. The granulosa cells were left to attach and luteinize for 24 h before non-attached cells and spent media were removed. Fresh media (100 μl) containing treatments were replaced every 24 h. Conditioned media were collected and frozen at −20°C for later determination of estrogen and progesterone concentrations by standard radioimmunoassay.

Preparation of treatments

Amitraz (Alltech, Sydney, Australia) in methanol (10 mg/ml) was diluted in F12/DMEM to make 1 mg/ml stock and filter-sterilized. Treatments of 1 (0.0001% w/v), 10 (0.001%), 50 (0.005%) and 100 (0.01%) μg/ml amitraz were made up by diluting an aliquot of 1 mg/ml stock in culture medium on the day of use. Appropriate methanol controls were used for each concentration of amitraz tested. For example, the 50 μg/ml amitraz controls were 1 : 200 dilutions of methanol in medium.

The α_2-AR antagonist yohimbine (Sigma, St Louis, MO, USA) was made into a 1 mol/l solution in water. This was diluted in F12/DMEM to make a 0.01 mol/l stock and filter-sterilized. Yohimbine treatments of 2, 1, 0.2, 0.02, 0.002 mmol/l were made up in culture medium on the day of use.

HCG (Chorulon; Intervet Australia, Bendigo, Australia) 1000 IU/ml stock was made up in solvent supplied by the manufacturer. This was diluted to 10 IU/ml with F12/DMEM, and frozen in 1 ml aliquots. Working hCG preparations of 1, 0.1, 0.01 and 0.001 IU/ml were made up in F12/DMEM from the frozen aliquots on the day of use.

Treatments

Luteinized human granulosa cells (10 000 per well) in quadruplicate from a total of 29 women were exposed to 1, 10, 50 and 100 μg/ml amitraz for 2, 4, 6, 24, 48 or 72 h. The numbers of granulosa cells collected from one woman were rarely enough to examine all 24 combinations of dose, time of exposure and the controls. Each treatment was therefore examined using granulosa cells from at least three women (n = 3). When additional cells were available, experiments were repeated to a maximum of n = 6 for any one treatment. Media were replenished at 24 h intervals. Media containing steroid hormones were collected, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assays were conducted on completion of specified exposures. Controls were the amitraz solvent methanol at the same dilution as the amitraz treatment. Each amitraz concentration was expressed as a percentage of its own methanol control for the relevant time point.

The effect of amitraz on hCG-stimulated steroidogenesis was determined by exposing luteinized granulosa cells in triplicate to 1, 10, 100, 1000 mIU/ml hCG ± 50 μg/ml amitraz for 6 h (n = 4). The control for hCG-only treatments was the manufacturer's hCG solvent diluted in F12/DMEM medium in the same manner as the 1000 mIU hCG treatment. This gave the same unstimulated basal progesterone and estrogen concentrations as medium alone. hCG-stimulated steroid hormone production was expressed as a percentage of the solvent control, such that identical steroid hormone production by both the hCG-treated and the control-treated cells would result in a value of 100%. Amitraz + hCG steroid hormone production was expressed as a percentage of the combined methanol + hCG solvent control.

The mechanism of action of amitraz was determined by exposing luteinized granulosa cells to 50 μg/ml amitraz for 6 h, ± 0.002, 0.02 0.2, mmol/l yohimbine (n = 5). The steroid hormone production per cell for each concentration of yohimbine was set as 100, and the corresponding amitraz + yohimbine treatment was expressed as a percentage of the yohimbine-only control.

MTT cell number assay

Metabolically active cells convert yellow, water-soluble MTT (Sigma) to the purple, water-insoluble end-product, formazan. The amount of formazan formed is proportional to the number of metabolically active cells (Mossman, 1983). Luteinized granulosa cells were incubated with 0.5 mg/ml MTT for 18 h, formazan crystals were extracted and dissolved by incubation with 20% sodium dodecyl sulphate in 0.02 mmol/l HCl for 1 h, and absorbance was measured at 570 nm in a Bio-Rad Plate Reader. Regents Park, NSW, Australia MTT standard curves were constructed by plating out granulosa cells at concentrations of 40 000 to 156 cells/well. Six replicates of the standard curve were constructed for the cells from each woman, and the numbers of granulosa cells remaining in wells after specified treatments were determined by comparison with the standard curve from the same woman. In some cases, the concentration of steroid hormones in a well was divided by the number of cells in the same well to give an approximation of the amount of hormone produced per cell. The mean of replicate hormone values per cell was calculated for each woman.

Estrogen and progesterone radioimmunoassay

Radioimmunoassay using human progesterone standards, rabbit anti-progesterone antiserum and ^{125}I-radiolabelled progesterone (all from ICN Pharmaceuticals, Costa Mesa, CA, USA) were used to quantify progesterone in tissue culture media. The interassay coefficient of variation was 14%, and the sensitivity of the assay was 0.2 ng/ml.

Similarly, estradiol (E_2) in tissue culture samples was quantified by radioimmunoassay using human E_2 standard (DSL, Webster, Texas, USA), rabbit anti- E_2-17β antiserum and ^{125}I-radiolabelled E_2 (ICN Pharmaceuticals). The cross-reactivity of the antiserum showed 100% cross-reactivity with E_2-17β and 6.5% or less with other steroids tested. The interassay coefficient of variation was 12% and the sensitivity of assay was 20 pg/ml.

Sheep anti rabbit serum (Silenus, Boronia, Australia) was used as a second antibody in both assays to separate bound and free fractions.

Statistical analysis

One-way analysis of variance was conducted on cell numbers, progesterone or estrogen values, and followed by Fisher's *post hoc* test. HCG data, expressed as percentages of controls, were subjected to χ^2 analysis, and a paired t-test was applied to data on progesterone per cell obtained after yohimbine treatment. Significance was assigned at $P < 0.05$. The data are presented as a percentage of control values in figures because of the amount of variability in basal steroidogenesis between patients. Points in figures represent the mean ± SEM of the percentage of controls from different patients (n refers to patient numbers).

Results

Amitraz was not cytotoxic to human luteinized granulosa cells at concentrations of 1, 10 or 50 μg/ml for exposures of 2–72 h. The highest concentration of amitraz tested, 100 μg/ml, caused significant cell death after exposures of 24, 48 and 72 h (Table 1). Amitraz tended to decrease the amount of progesterone produced by each granulosa cell during exposure periods of 2, 4 and 6 h (Figure 1), but high biological variation (Table 2) obscured the results. Progesterone per cell production in control wells was 0.00766 ± 0.00497 ng/ml (mean ± SD, Table 2) after 2 h, which suggests a production rate of 0.003 ng/ml per cell per hour. The hourly production rates were also 0.003 ng/ml per cell for both the 4 and 6 h control incubation times, suggesting that progesterone production per cell was linear over the 6 h range examined.

Table I. Effects of amitraz on luteinized granulosa cell viability

Hours of exposure	Amitraz (μg/ml)							
	1		10		50		100	
	n[a]	% of control[b]	n[a]	% of control[b]	n[a]	% of control[b]	n[a]	% of control[b]
2	3	111 ± 2	3	108 ± 19	3	102 ± 11	5	90 ± 8
4	4	106 ± 5	4	96.4 ± 11	4	105 ± 15	6	101 ± 26
6	4	96.9 ± 7	4	129 ± 50	10	101 ± 10	4	91.6 ± 10
24	3	91.4 ± 4	6	92.5 ± 16	3	89 ± 16	4	50.6 ± 14**
48	3	92.4 ± 7	5	111 ± 22			4	26.5 ± 12*
72	3	86.7 ± 3	3	71 ± 45			3	38.2 ± 12*

Luteinized human granulosa cells were exposed to amitraz (1, 10, 50 and 100 μg/ml) for 2, 4, 6, 24, 48 and 72 h and the numbers of viable cells remaining at the end of the exposure periods were determined in a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay.
[a]Number of times the experiment was repeated with cells from different women.
[b]Calculated by expressing the viable cell number per well after amitraz treatment as a percentage of the methanol control; mean ± SD of n percentages.
Cell numbers (treated versus controls) were subjected to ANOVA.
*$P < 0.05$; **$P < 0.01$.

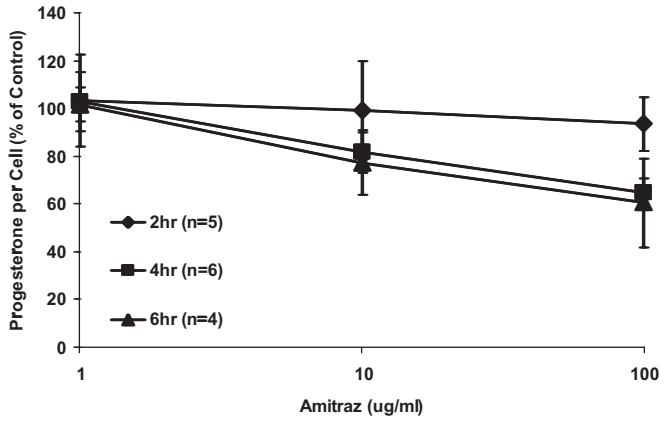

Figure 1. Effect of short amitraz exposures on progesterone production per cell. Human luteinized granulosa cells were exposed to 1, 10 and 100 μg/ml amitraz for 2, 4 and 6 h. Progesterone (ng/ml) secreted into culture media was measured by radioimmunoassay. Cell numbers were determined by the MTT assay. Progesterone production per cell was determined, subjected to analysis of variance, then expressed as a percentage (mean ± SEM) of the relevant control.

Effect of 1 μg/ml (0.0001% w/v) amitraz on human luteinized granulosa cells

Exposures from 2 to 72 h had no significant effect upon cell number (Table 1), progesterone (Figures 1 and 3) or estrogen production (Figure 2) by human luteinized granulosa cells.

Effect of 10 μg/ml (0.001% w/v) amitraz on human luteinized granulosa cells

Exposure from 2 to 72 h did not significantly reduce cell numbers (Table I) or estrogen production (Figure 2). Two hours of exposure to 10 μg/ml amitraz did not affect progesterone production, but 4 and 6 h of exposure tended to reduce progesterone production per cell to 82 ± 4% (mean ± SEM, $n = 6$) and 77 ± 7% (mean ± SEM, $n = 4$) of methanol control values respectively (Figure 1). Longer exposures to 10 μg/ml amitraz significantly decreased progesterone production to 58 ± 8% ($P < 0.01$, $n = 6$) of control values after 24 h exposure, to 58 ± 3% ($P < 0.05$, $n = 5$) after 48 h exposure (data not shown) and to 63 ± 8% ($P < 0.05$, $n = 3$) after 72 h exposure (Figure 3).

Effect of 50 μg/ml amitraz on human luteinized granulosa cells

Exposure for 6 h did not affect cell numbers, which were 101 ± 10% of methanol control values (mean ± SD, $n = 10$, Table I), or estrogen production, which was 97 ± 14% of control values (mean ± SEM, $n = 4$). Progesterone production (mean ± SEM) by luteinized granulosa cells exposed to 50 μg/ml amitraz was 89 ± 21% of methanol control values for 2 h of exposure ($n = 5$), 85 ± 8% for 4 h of exposure ($n = 6$) and 82 ± 6% for 6 h of exposure ($n = 4$). The 50 μg/ml dose of amitraz inhibited basal progesterone production by approximately 15% at exposures of 2–6 h. There was no significant difference between the 50 μg/ml amitraz methanol control and F12/DMEM media; cell numbers were 13209 ± 6088 and 13968 ± 5941, and progesterone production was 148 ± 4 and 169 ± 63 ng/ml respectively (mean ± SD, $n = 6$).

Effect of 100 μg/ml (0.01% w/v) amitraz on human luteinized granulosa cells

Two hours of exposure to 100 μg/ml amitraz had no effect on progesterone production by granulosa cells obtained from four out of five women, but exposures of 4 and 6 h reduced progesterone per cell production to 64.8 ± 13.5% and 60.47 ± 31.8% (mean ± SD) of time-matched controls respectively (Table II and Figure 1). Significant cell death caused by high amitraz concentrations (100 μg/ml; Table I) resulted in reduced estrogen ($P < 0.01$) and progesterone ($P < 0.05$ and $P < 0.01$) production for the corresponding treatments (Figures 2 and 3) when compared with controls.

Effect of amitraz on HCG-stimulated hormone production by human luteinized granulosa cells

Exposure to HCG for 6 h stimulated both estrogen and progesterone production in a dose-dependent fashion (Figures 4 and 5). A paired t-test on 0 HCG vs 1000 HCG estrogen concentrations was significantly different ($P < 0.001$, $n = 4$)) HCG significantly stimulated estrogen production after a 6 h exposure. Interestingly, exposure to 50 μg/ml amitraz for 6 h did not affect HCG-stimulated

Table II. Effects of amitraz on progesterone production by luteinized granulosa cells

Woman	Exposure time								
	2h			4 h			6 h		
	Control	Amitraz	%	Control	Amitraz	%	Control	Amitraz	%
1	0.011	0.011	99	0.022	0.015	70	0.018	0.017	94
2	0.004	0.004	102	0.005	0.003	66	0.013	0.006	45
3				0.072	0.060	83	0.043	0.034	79
4	0.001	0.0009	92	0.001	0.0008	50	0.0025	0.0005	24
5	0.013	0.0075	56	0.023	0.017	48			
6	0.009	0.010	117	0.012	0.008	72			
Mean	0.008	0.007	93	0.0126	0.0087	65	0.0187	0.0143	60
SD	0.005	0.004	23	0.0098	0.0071	14	0.0177	0.0147	32

Luteinized human granulosa cells were exposed to amitraz (100 μg/ml) for 2, 4 and 6 h. Progesterone per well (ng/ml) was divided by the number of cells in that well, and the mean of four replicate wells obtained for each woman. Control values were obtained from cells cultured with standard culture medium containing methanol at the same dilution as cells in the amitraz-treated wells. The effect of amitraz on progesterone production per cell was expressed as a percentage of the control for each woman. There were no significant differences between treatments at any time-point using ANOVA.

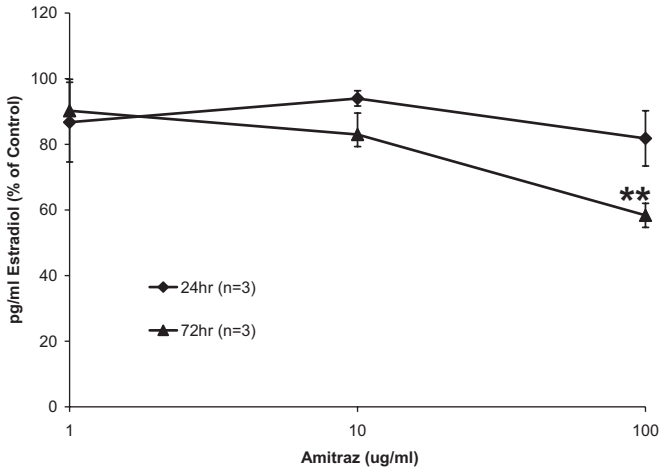

Figure 2. Effect of amitraz on estrogen production. Human luteinized granulosa cells were exposed to 1 (n = 3), 10 (24 h, n = 6; 72 h, n = 3) and 100 μg/ml (24 h, n = 4; 72 h, n = 3) amitraz for 24 and 72 h. Estrogen secreted into the culture medium was measured by radioimmunoassay and subjected to ANOVA. Estrogen (pg/ml) is shown as a percentage (mean ± SEM) of the relevant control. $^{**}P < 0.01$.

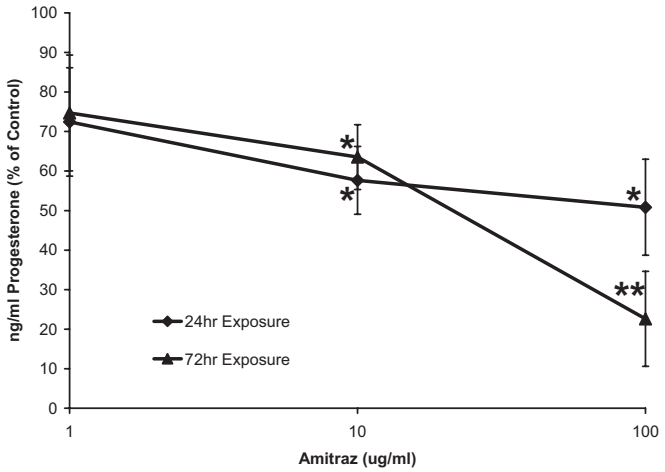

Figure 3. Effect of long amitraz exposures on progesterone production. Human luteinized granulosa cells were exposed to 1 (n = 3), 10 (24 h, n = 6; 72 h, n = 3) and 100 μg/ml (24 h, n = 4 72 h, n = 3) amitraz for 24 and 72 h. Progesterone secreted into the culture medium was measured by radioimmunoassay and subjected to ANOVA. Progesterone (ng/ml) is shown as a percentage (mean ± SEM) of the relevant control.$^{*}P < 0.05$; $^{**}P < 0.01$.

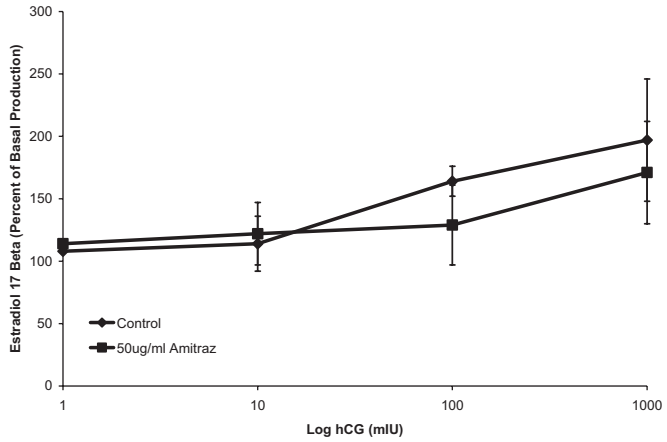

Figure 4. Effect of amitraz on HCG-stimulated estrogen production. Human luteinized granulosa cells were exposed to 1, 10, 100 or 1000 mIU HCG, or to one of these HCG doses and 50 μg/ml amitraz, for 6 h. HCG treatments are expressed as a percentage of the HCG solvent control; HCG + amitraz treatments are expressed as a percentage of the HCG solvent + methanol control. All percentages (for four women) are expressed as mean ± SEM.

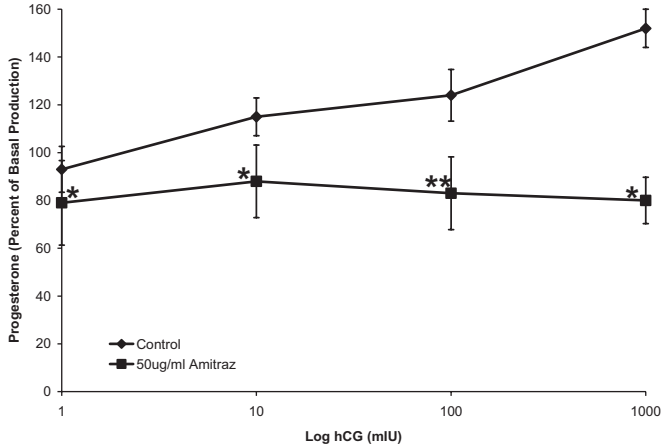

Figure 5. Effect of amitraz on HCG-stimulated progesterone production. Human luteinized granulosa cells were exposed to 1, 10, 100 or 1000 mIU HCG, or to one of these HCG doses + 50 μg/ml amitraz, for 6 h. HCG treatments are expressed as a percentage of the HCG solvent control; HCG + amitraz treatments expressed as a percentage of the HCG solvent + methanol control. All percentages (for four women) are expressed as mean ± SEM. Percentages for amitraz + HCG are compared with percentages for the same dose of HCG in a χ^2 analysis. $^{*}P < 0.05$; $^{**}P < 0.01$.

estrogen production (Figure 4), but did prevent HCG stimulation of progesterone production in the presence of all concentrations of HCG (Figure 5). In addition, the 50 μg/ml dose of amitraz further reduced HCG-stimulated progesterone production to levels that were approximately 15% lower than basal, unstimulated control concentrations.

Effect of yohimbine on amitraz-mediated inhibition of progesterone production

The α_2-AR antagonist, yohimbine, had no effect on progesterone production. Progesterone per cell values were 91 ± 5, 99.7 ± 14 and 116 ± 16% (mean ± SD) of control progesterone per cell values after yohimbine treatments of 0.002, 0.02 and 0.2 mmol/l respectively. Exposure to 50 μg/ml amitraz for 6 h caused progesterone production per cell to be 0.0126 ± 0.004 ng/ml (mean ± SEM, n = 5), whereas exposure to 50 μg/ml amitraz + 0.2 mmol/l yohimbine resulted in a significant increase ($P < 0.01$) in progesterone production per cell to 0.0225 ± 0.007 ng/ml; 0.2 mmol/l yohimbine prevented amitraz-mediated inhibition of progesterone production. Progesterone production per cell after 6 h exposure to 0.2 mmol/l yohimbine was 0.0225 ± 0.007 ng/ml, exactly the same as the amitraz + yohimbine treatment although the composite data

from individual women were not identical. Progesterone per cell from each amitraz + yohimbine treatment was expressed as a percentage of the corresponding yohimbine treatment from the same woman. The 0.2 mmol/l yohimbine + amitraz treatment was 99.4 ± 2% (mean ± SD, n = 5; Figure 6) of the yohimbine treatment. Reduction in yohimbine concentration resulted in decreased progesterone production per cell in the presence of 50 μg/ml amitraz (Figure 6).

Discussion

Exposure to 1 μg/ml (0.0001%) amitraz did not cause cytotoxicity or reduce steroidogenesis in human luteinized granulosa cells treated for periods of 2–72 h. The higher concentration of 10 μg/ml (0.001%) amitraz similarly did not cause cell death, but was found to inhibit progesterone production whilst having no effect upon estrogen production. Amitraz decreased the amount of progesterone produced by each cell after a 4 h exposure; the reduction in progesterone concentration was not caused by decreased cell viability. 24h exposure to 100 μg/ml (0.01%) amitraz was cytotoxic, but still had the same effect on steroidogenesis as that noted at lower doses and exposures; progesterone production was significantly reduced. Exposure to the highest dose of amitraz for 72 h also caused cell death, but at this exposure the reduction in cell number was accompanied by reduced production of both estrogen and progesterone.

How could progesterone but not estrogen synthesis be differentially inhibited? Cholesterol is converted to pregnenolone by the cytochrome P450 side-chain cleavage enzyme, and pregnenolone is converted to progesterone by 3β-hydroxysteroid dehydrogenase (3βHSD). Progesterone can be a precursor for estrogen synthesis, but if this were the case, the amitraz-mediated inhibition of progesterone production would reduce the amount of precursor available for estrogen synthesis with a consequent reduction in estrogen production. Pregnenolone is also a precursor for estrogen synthesis. The amount of pregnenolone converted to progesterone is dependent upon the activity of only one enzyme; 3βHSD. Therefore we speculate that amitraz reduces progesterone, but not estrogen synthesis, by inhibiting 3βHSD activity. Another possibility is that amitraz increased the metabolism of progesterone. If this were the case, the doses of amitraz examined were insufficient to cause complete metabolism of all the progesterone that was produced.

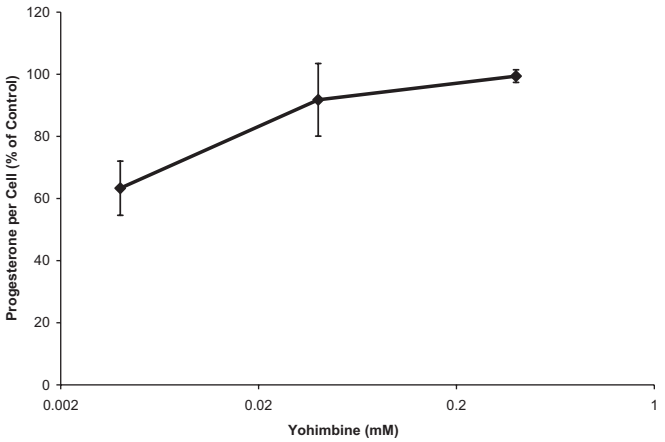

Figure 6. Effect of yohimbine on amitraz-mediated inhibition of progesterone production. Human luteinized granulosa cells were exposed to 50 μg/ml amitraz ± 0.002, 0.02 or 0.2 mmol/l yohimbine for 6 h. Progesterone (ng/ml) secreted into the culture medium was measured by radioimmunoassay. Cell numbers were determined by MTT assay. Progesterone production per cell during amitraz + yohimbine treatment was expressed as a percentage of yohimbine-only controls; 100% means that yohimbine completely blocked the effects of amitraz. All percentages (five women) are expressed as mean ± SEM.

The magnitude of HCG-stimulated steroidogenesis was consistent with previous reports (Endo *et al.*, 1993). Amitraz abolished the stimulatory effects of HCG on progesterone production, but had no effect on HCG-stimulated estrogen production, lending support to the speculation that amitraz blocked the action of HCG by acting on 3βHSD. It was found that 50 μg/ml amitraz caused basal progesterone production to be reduced by approximately 15%, and this could be attributed to a 15% increase in progesterone metabolism. However, if this were the case, it would be expected that HCG-stimulated progesterone production would only be reduced by 15%, thus giving rise to an attenuated HCG response. Since HCG stimulation was abolished, and not just reduced, it was concluded that amitraz did not cause metabolism of progesterone and instead reduced progesterone production by another mechanism.

The levels of 3βHSD mRNA in primate corpora lutea are highest during the early luteal phase, and decrease as the luteal phase proceeds (Fairchild Benyo *et al.*, 1993; Duncan *et al.*, 1999). In primates, administration of either of the luteolytic agents prostaglandin $F_2\alpha$ or GnRH antagonist caused a decrease in 3βHSD mRNA and protein within 24 h and so prevented LH-stimulated steroidogenesis (Fairchild Benyo *et al.*, 1993; Duncan *et al.*, 1998). In the present study, amitraz abolished HCG-stimulated progesterone, but not estrogen production within 6 h, which suggests that amitraz acted distally to the production of pregnenolone, and blocked HCG stimulation by down-regulating 3βHSD. It is possible that amitraz may activate existing physiological luteolytic mechanisms that down-regulate 3βHSD.

Yohimbine is an α_2-AR antagonist (Lalchandani *et al.*, 2002) that prevented the action of amitraz in other systems (Altobelli *et al.*, 2001). In this study, yohimbine prevented amitraz-mediated inhibition of progesterone production in a dose-dependent fashion, suggesting firstly that human luteinized granulosa cells express α_2-ARs, and secondly that α_2-ARs mediated the mechanism of action of amitraz. A 6 h exposure to the α-AR agonist phenolephrine (PHE) did not affect basal progesterone production, but did inhibit HCG-stimulated progesterone production (Fohr *et al.*, 1993). These data are consistent with those in this report. Amitraz is an α-AR agonist which did not cause a statistically significant reduction in basal progesterone production during a 6 h exposure, but did cause a significant inhibition of HCG-stimulated progesterone production in the same time period. In addition, PHE alone had no effect on cytosolic free Ca^{2+} levels, but the combination of HCG and PHE elevated Ca^{2+} (Fohr *et al.*, 1993). It is not known if a 24 h exposure to PHE alone would have inhibited progesterone production in a manner similar to amitraz, or if

the combination of amitraz and HCG would elevate Ca^{2+} in the same manner as PHE + HCG. The effect of elevated intracellular Ca^{2+} on 3βHSD activity is also unknown. Both amitraz and PHE bind α-ARs. Noradrenalin is a physiological ligand for both α and β-ARs, but the β-AR agonist isoproteronol had no effect on HCG-stimulated progesterone production or cytosolic Ca^{2+} levels in human luteinized granulosa cells, suggesting that only the α-AR mediates inhibition of progesterone production (Fohr *et al.*, 1993). Noradrenalin slightly reduced progesterone production by four human corpora lutea *in vitro* (Casper and Cotterell, 1984), which was consistent with the finding that amitraz, a noradrenalin analogue, inhibited progesterone production by human granulosa-lutein cells, one of the components of the human corpus luteum.

The amitraz in cattle dips and dog collars is commonly used at concentrations of 0.025%. The dermal absorption of amitraz by humans can cause hyperglycaemia and have other physiological effects (Kalyoncu *et al.*, 2002), but blood concentrations of amitraz have not been correlated to the area or duration of dermal exposure. Metabolism and clearance rates of amitraz in humans are not clear, and it is not known if amitraz in the peripheral blood system would cross the basement membrane of ovarian follicles and gain access to granulosa cells. However, the corpus luteum is highly vascularized, and it is possible that amitraz in circulating blood could gain access to α_2-ARs expressed by granulosa-lutein cells *in vivo*.

If amitraz was found to also reduce progesterone production by women *in vivo*, would there be any impact on fertility? The amount of progesterone that a woman synthesizes can be represented by a 'progesterone index' (PI), which can be determined by calculating the mean of four progesterone measurements taken daily during the early luteal phase. It was found that the PI remains constant between cycles for any one woman, and that women with PI lower than 30 nmol/l have difficulty becoming pregnant (Lenton and Landgren, 1985; Sallam *et al.*, 1999). If a woman already has a low PI, then exposure to amitraz has the potential to exacerbate the situation and decrease progesterone production to levels associated with infertility. Additionally, this study found that a 6 h exposure to 50 μg/ml (0.005%) amitraz completely abolished the embryonic HCG signal. Exposure to amitraz that results in blood concentrations of >50 μg/ml for 6 h therefore has the potential to prevent establishment of pregnancy or to cause miscarriage. However, the relative importance of amitraz's inhibition of luteal progesterone production with respect to overall fertility has yet to be elucidated, although amitraz is also reproductively toxic in the hypothalamus (Altobelli *et al.*, 2001), where its action ultimately prevents ovulation in females (Goldman and Cooper, 1993), and probably reduces sperm production in males (Al-Thani *et al.*, 2003). This is the first report of amitraz having an effect on human cells. Since amitraz activated α_2-ARs expressed by human luteinized granulosa cells, amitraz may also bind to hypothalamic α_2-ARs, with the potential to prevent the ovulatory LH surge in women. Amitraz can be absorbed through the skin and have systemic effects in humans (Kalyoncu *et al.*, 2002). Taken together, these data suggest that amitraz has the potential to be a reproductive toxin in humans.

In conclusion, amitraz bound to α_2-ARs expressed by human luteinized granulosa cells and inhibited basal progesterone, but not estrogen, production. Amitraz also inhibited HCG-stimulated progesterone production, but not estrogen production. This differential inhibition of progesterone production but not estrogen production suggests that amitraz may act on the steroidogenic enzyme 3βHSD. The lowest dose of amitraz tested, 1 μg/ml (0.0001%), had no effect when cells were exposed for 2–72 h, but 10 μg/ml (0.001%) inhibited progesterone production and 100 μg/ml (0.01%) amitraz killed cells after a 24 h exposure. The reproductive toxicity of amitraz to mammals has already been established (Goldman and Cooper, 1993), but this is the first study to show that amitraz inhibits the production of the steroid hormone progesterone, directly, and that amitraz is toxic to human reproductive cells in an *in vitro* model system.

Acknowledgements

The authors wish to acknowledge the patients of the Flinders Reproductive Medicine for the donation of granulosa cells, and to thank the Unit staff for informing patients, obtaining their consent, and for their technical assistance in the collection of human granulosa cells. Louise Brussow originally suggested amitraz for investigation and started work on the development of the granulosa cell isolation procedures under the supervision of Dr Tina Lavranos. This research was supported by a grant from the Flinders Medical Centre Foundation.

References

Abu-Basha EA, Yibchok-Anun S, Hopper DL and Hsu WH (1999) Effects of the pesticide amitraz and its metabolite BTS 27271 on insulin and glucagon secretion from the perfused rat pancreas: involvement of alpha2D-adrenergic receptors. Metab Clin Exp 48,1461–1469.

Al-Thani AS, Al-Thani A, Elbetieha A and Darmani H (2003) Assessment of reproductive and fertility effects of amitraz pesticide in male mice. Toxicol Lett 138,235–260.

Altobelli D, Martire M, Maurizi S and Preziosi P (2001) Interaction of formamidine pesticides with the presynaptic alpha(2)-adrenoceptor regulating. Toxicol Appl Pharmacol 172,179–185.

Bodis J, Tinneberg HR, Torok A, Cledon P, Hanf V and Papenfuss F (1993) Effect of noradrenaline and dopamine on progesterone and estradiol secretion of human granulosa cells. Acta Endocrinol 129,165–168.

Casper RF and Cotterell BA (1984) The effects of adrenergic and cholinergic agents on progesterone production by human corpus luteum in vitro. Am J Obstet Gynecol 148,663–669.

Chen TH and Hsu WH (1994) Inhibition of insulin release by a formamidine pesticide amitraz and its metabolites in a rat beta-cell line: an action mediated by alpha-2 adrenoceptors, a GTP-binding protein and a decrease in cyclic AMP. J Pharmacol Exp Ther 271,1240–1245.

Diaz F, Anderson L, Wu Y, Rabot A, Tsai S and Wiltbank M (2002) Regulation of progesterone and prostaglandin F2 alpha production in the CL. Mol Cell Endocrinol 191,65–80.

Duncan WC, Illingworth PJ, Young FM and Fraser HM (1998) Induced luteolysis in the primate: rapid loss of luteinising hormone receptors. Hum Reprod 13,2532–2540.

Duncan WC, Cowen GM and Ilingworth PJ (1999) Steroidogenic enzyme expression in human corpora lutea in the presence and absence of exogenous human chorionic gonadotrophin (HCG) Mol Hum Reprod 5,291–298.

Eamens G, Spence S and Turner M (2001) Survival of Mycobacterium avium subsp paratuberculosis in amitraz cattle dip fluid. Aust Vet J 79,703–706.

Elfassy O, Goodman F, Levy S and Carter L (2001) Efficacy of an amitraz-impregnated collar in preventing transmission of Borrelia burgdorferi by adult Ixodes scapularis to dogs. J Am Vet Med Assoc 219,185–189.

Endo T, Aten R, Leykin L and Behrman HR (1993) Hydrogen peroxide evokes antisteroidogeneic and antigonadotrophic actions in human granulosa luteal cells. J Clin Endocrinol Metab 76,337–342.

Fairchild Benyo D, Ravindranath N, Bassett S, Hutchison J and Zeleznik AJ (1993) Cellular aspects of corpus luteum function in the primate. Hum Reprod 8,102–106.

Fohr KJ, Mayerhofer A, Sterzik K, Rudolf M, Rosenbusch B and Gratzl M (1993) Concerted action of human chorionic gonadotrophin and norepinephrine on intracellular-free calcium in human granulosa-lutein cells: evidence for the presence of a functional alpha-adrenergic receptor. J Clin Endocrinol Metab 76,367–373.

Goldman JM and Cooper RL (1993) Assessment of toxicant-induced alterations in the luteinizing hormone control of ovulation in the rat. In: Chapin H, ed. Female reproductive toxicology. San Diego, CA: Academic Press, pp.79–91.

Gosselin RE, Smith RP, Hodge HC (1984) Clinical toxicology of commercial products. 5th edn. Baltimore, MD: Williams and Wilkins.

Gunaratnam P, Wilkinson G and Seawright A (1983) A study of amitraz toxicity in cats. Aust Vet J 60,278–279.

Hollingworth R (1976) Chemistry, biological activity, and uses of formamidine pesticides. Environ Health Perspect 14,57–69.

Hsu WH and Schaffer DD (1988) Effects of topical application of amitraz on plasma glucose and insulin concentrations in dogs. Am J Vet Res 49,130–131.

Kalyoncu M, Dilber E and Okten A (2002) Amitraz intoxication in children in the rural Black Sea region: analysis of forty-three patients. Hum Exp Toxicol 21,269–272.

Lalchandani SG, Longping L, Zheng W, Suni MM, Moore RM, Liggett SB, Miller DD and Feller DR (2002) Yohimbine dimers exhibiting selectivity for the human alpha2c-adrenoreceptor subtype. J Pharmacol Exp Ther 303,979–984.

Lenton E and Landgren B (1985) The normal menstrual cycle. In: Shearman R, ed. Clinical reproductive endocrinology. Churchill Livingstone, London. pp. 81–107.

McDougall K and Lewis I (1984) Behaviour of amitraz in cattle dipping baths. Aust Vet J 61,137–140.

Mekonnen S (2001) In vivo evaluation of amitraz against ticks under field conditions in Ethiopia. J S Afr Vet Assoc 72,44–45.

Mossman T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65,55–63.

Paradis M (1999) New approaches to the treatment of canine demodicosis. [Review]. Vet Clin North Am Small Anim Pract 29,1425–1436.

Pass MA and Mogg TD (1991) Effect of amitraz and its metabolites on intestinal motility. Comp Biochem Physiol C Comp Pharmacol Toxicol 99,169–172.

US Environmental Protection Agency (1996) Reregistration Eligibility Decision for Amitraz. EPA-738-F-96–031.

Sallam H, Sallam A, Ezzeldin F, Agamia A and Abou-Ali A (1999) Reference values for the midluteal plasma progesterone concentration: evidence from human menopausal gonadotrophin-stimulated pregnancy cycles. Fertil Steril 71,711–714.

Schaub L, Sardy S and Capkun G (2002) Natural variation in baseline data: when do we call a new sample 'resistant'? Pest Manage Sci 58,959–963.

Shaw S and Foster A (2000) Treatment of canine adult-onset demodicosis. Aust Vet J 78,243–244.

Weichel L and Nauen R (2003) Monitoring of insecticide resistance in damson hop aphid Phorodon humuli Schrank (Hemiptera: Aphididae) from German hop gardens. Pest Manage Sci 59,991–998.

Yim G, Holsapple M, Pfister W and Hollingworth R (1978) Prostaglandin synthesis inhibited by formamidine pesticides. Life Sci 23,2509–2516.

Submitted March 3, 2005; resubmitted May 19, 2005; accepted June 1, 2005