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The mammary tumor response in triazine-treated female rats: A threshold-mediated interaction with strain and species-specific reproductive senescence

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Abstract

Triazine herbicides are among the most heavily used agricultural pesticides. Although they possess a very low acute toxicity in animals, a mammary tumor response has been consistently observed in Sprague-Dawley (SD) female rats following chronic oral dosing of atrazine and simazine at and above maximum tolerated doses. However, a substantial collection of detailed research has clearly shown that triazines are not genotoxic or mutagenic, nor do they possess estrogenic agonist activity that might promote mammary tumor growth. Examination of estrous cycling records of atrazine-treated SD rats revealed a premature appearance of persistent estrous episodes, beyond the prevalent occurrence normally seen in untreated, aging SD rats. A significant correlation has been found between early or severe estrous cycle disruption of atrazine-treated rats and the early appearance of mammary tumors. In studies using SD female rats fed atrazine for 6 months, then ovariectomized and administered an estrogen-containing silastic s.c. implant, a deficient luteinizing hormone surge was observed at a 400 parts per million (ppm) dose, but not at 25 or 50 ppm. Because SD rats exhibiting persistent estrus also have a prolonged elevation of estrogen secretion, it is proposed that the triazine-associated mammary tumor response is promoted by the test animal's own estrogen from ovarian follicles that fail to ovulate because gonadotropin surge sufficiency is blocked by the high dose of herbicide. It is further proposed that, because reproductive senescence in SD rats is fundamentally different from menopause in women, the animal response to dosing, as well as the enormous requisite dosing level, establishes a safety margin of very low risk to human health from this mode of action.

Keywords: Atrazine; Simazine; Mammary tumors; Luteinizing hormone (LH); Estrous cycle

1. Introduction

Triazine herbicides are used in agriculture to control growth of annual grasses and broadleaf weeds. The principal forms, atrazine and simazine (Fig. 1), have been applied for many years around crops of corn, sorghum, and Christmas trees. Triazines inhibit photosynthesis in plants by preventing electron transfer at the reducing site of chloroplast complex II [1], and thus correspondingly demonstrate only low-level toxicity to non-photosynthetic organisms. For example, the oral LD_{50} of atrazine in rodents is 3000 mg/kg, and the maximum-tolerated dose (MTD) in chronically fed rats is about 40 mg/kg [2], both of which are 10 000 times greater than the IC_{50} in chloroplasts.

Atrazine and simazine have been extensively examined in a variety of in vivo toxicologic screens, and no observed effect levels (NOELS) for chronic toxicity are very high ($\geq$70 parts per million, or ppm) in rats, mice, and dogs [3]. A review of other pertinent studies is also found in the Hauswirth and Wetzel reference [3]: NOELS for reproductive and developmental toxicity of 5 mg/kg/day in New Zealand White rabbits and 25 mg/kg/day in Sprague-Dawley rats, and a 2-generation feeding study in SD rats that identified a NOEL of 5 mg/kg/day. The review concludes that triazines pose no reproductive or developmental hazard in animals. Triazines have also been assessed in more than 40 mutagenicity-genotoxicity tests using in vivo markers as well as prokaryotic and eukaryotic cells [4–6], and a complete weight-of-evidence analysis concluded that the herbicides are neither genotoxic nor mutagenic [6].

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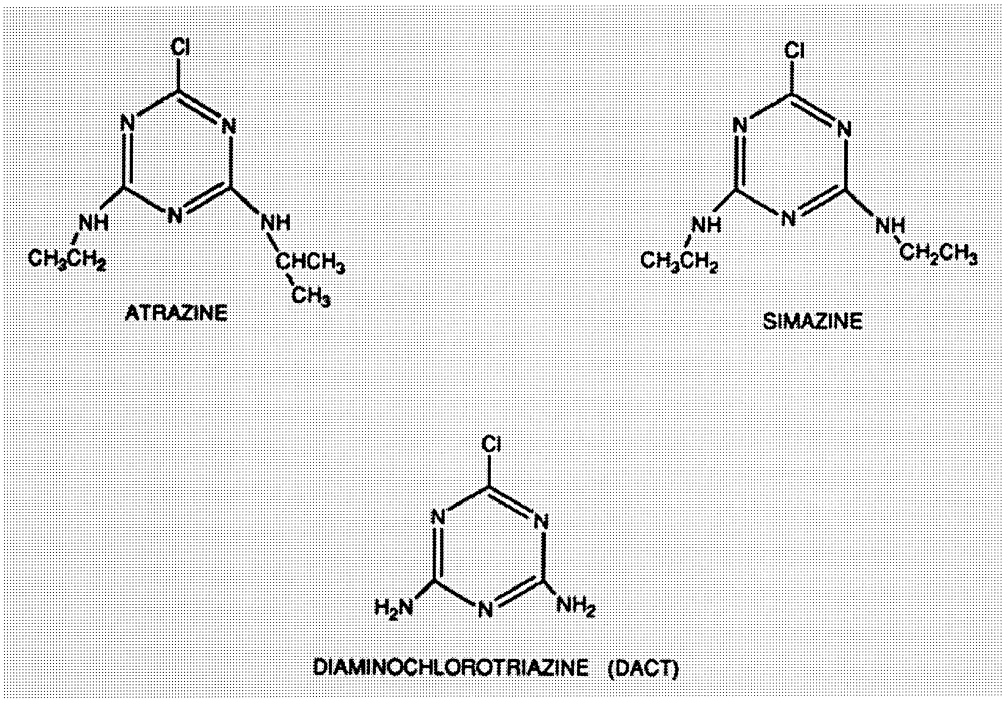

Fig. 1. Chemical structures of atrazine, simazine and a diaminochlorotriazine, a metabolite of atrazine and simazine.

2. Description of the problem

In chronic feeding studies with female SD rats, an increased incidence, and/or earlier appearance, of mammary tumors has been associated with atrazine dosing at high levels (Table 1). Of five 2-year studies, three showed a significant dose-related increase in final incidence of animals with mammary fibroadenomas, adenocarcinomas, or both, one study demonstrated an earlier, but not final, dose-related increase, and one study showed no effect of dosing (Table 1). The significant results usually occurred at or above the MTD range of 400 ppm, and only rarely at lower feeding levels.

There is further inconsistency of the response in that it failed to appear in Fischer-344 female rats given equally high doses of atrazine, nor was it seen in atrazine-treated

Table 1
Mammary tumor incidence rates in five 2-year dosing studies with atrazine in female Sprague-Dawley rats

DOSE (ppm)	0	10	25	50	70	100	400	500	1000
Adenocarcinoma	11/54	8/52				12/54			13/49
	15/88	16/69			27/69*			27/70*	43/89*
	17/60				13/59		22/60		
	8/30	4/40		5/40				6/29	
	12/80		18/80	20/78*	14/80		27/80*		
Percent	20.2	17.4	22.5	21.2	26.0	22.2	35.0	33.3	40.6
Fibroadenoma	11/54	20/52				14/54			22/49*
	29/88	29/69			36/69			39/70	45/89*
	39/60				30/59		41/60		
	4/30	6/40		10/40				8/29	
	16/80		25/80	33/78*	29/80*		25/80*		
Percent	31.7	34.2	31.3	36.4	45.7	25.9	47.1	47.5	48.6
Animals with any mammary tumor	22/54	28/52				26/54			35/49
	35/88	40/69			48/69			48/70*	65/89*
	46/60				34/59		49/60		
	11/30	10/40		13/40				11/29	
	24/80		34/80	44/78	38/80		43/80		
Percent	44.2	48.4	42.5	48.3	57.7	48.2	65.7	59.6	72.5

* Significantly different from 0 ppm incidence, $P < 0.05$. Compiled from Refs. [2,7,9].

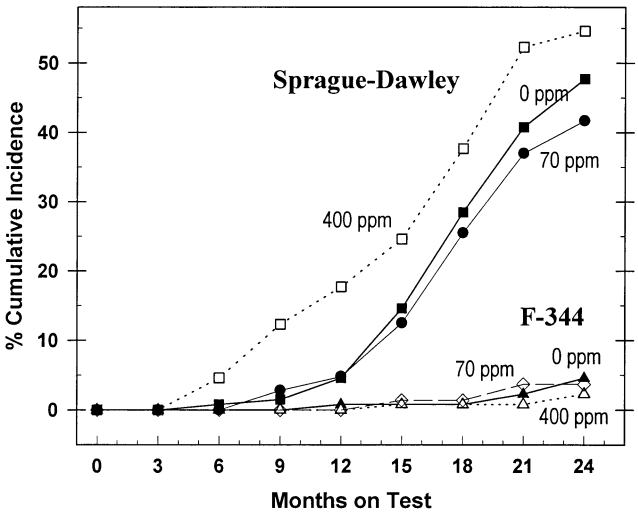

Fig. 2. Percent incidence of mammary tumors in adult Sprague-Dawley or Fischer-344 female rats fed atrazine for 24 months. Treatment as a dietary supplement was begun at 6–7 weeks of age and the study was terminated after 102 weeks of dosing. Tumors were proven at necropsy. Sixty animals/group (from Refs. [2,9]).

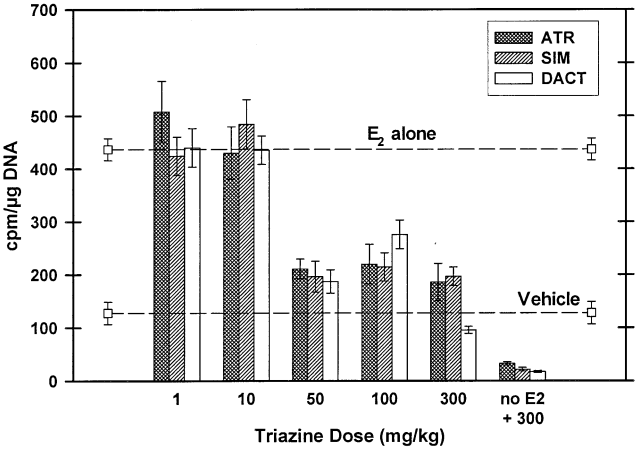

Fig. 3. Incorporation of [^{3}H]thymidine into uterine DNA in vitro in immature ovariectomized rats treated with triazine herbicides and estradiol. Animals were administered the indicated doses for 2 consecutive days and were injected once s.c. with 0.15 µg estradiol on Day 2 of triazine treatment. On the following day the uteri were dissected, and tissue slices were incubated with [^{3}H]thymidine in 95% O_2/5% CO_2, at 37°C, for 60 min. The slices were homogenized and extracted with perchloric acid, aliquots were counted by liquid scintillation and DNA was quantified by the diphenylamine reaction. Histograms represent means ± S.E. of 8–26 animals. Additional procedural details are found in Ref. [18].

male rats of either the SD or F344 strains, nor in atrazine-treated ovariectomized SD rats [7]. Four studies of oral atrazine administration have also been conducted on three strains of mice: (C57BL/6 × 3CH/Anf)F_1 [8], (C57BL/6 × AKR)F_1 [8], and CD-1 twice [7]. All produced negative results at chronic feeding doses up to 3000 ppm.

As discussed in depth by Stevens et al. [7], some important general conclusions have been drawn from these rodent studies: First, positive mammary tumor responses occurred only in an animal model with a normally high spontaneous background incidence (see Table 1: the average untreated tumor incidence, at 2 years of age, was 44.2%). Second, the tumor response was observed predominately in old age, and a large number of even high-dose animals did not develop tumors at all (direct-acting carcinogens normally produce tumors within a few weeks of a single administration, and virtually every animal responds). Third, histopathologic examination of the tumors showed that they were qualitatively identical in treated and untreated rats; no new pathology appeared with atrazine treatment. Fourth, records of initial palpation from one 2-year study [2,9] showed an earlier incidence of developing tumors but not a final higher incidence. A graph of this cumulative incidence is shown in Fig. 2. Fifth, responses usually occur only at very high oral doses, approaching or exceeding the MTD.

Because triazines were also determined to be not genotoxic or mutagenic, and because the treatment-associated tumor responses occurred only in one sex of one strain of a species highly prone to the same type of tumors, it was concluded that the treatment-associated results may be closely tied to *normal and spontaneous* pathophysiologic events of the SD rat strain. In other words, if normal aging predisposes mammary tumor development in the SD strain, then the addition of high-dose atrazine might manipulate the *rate of developing pathology*, rather than produce a new pathology. This conclusion would also explain the negative results in F344 female rats, ovariectomized rats, male rats and mice, because these models do not have a high spontaneous tumor incidence capable of being manipulated. Additional studies have been conducted to test the hypothesis that high-dose triazine administration facilitates aspects of reproductive senescence in SD female rats, and examples of results are presented here.

3. Tests of estrogen-related activity

Because rodent mammary tumors are typically hormone-dependent, and the presence of estrogens and/or prolactin has been repeatedly shown to promote tumor growth [10–12], it was important to assess the ability of triazine herbicides to act as estrogen agonists. Indeed, a number of other chlorinated hydrocarbons, including some active pesticides, do possess an identified estrogen agonist activity [13–17].

Results from a number of studies on estrogenic activity have now been published, including some from our laboratory, with the clear demonstration that triazines have no intrinsic estrogenic activity. In the example shown in Fig. 3, the expected estrogen-stimulated incorporation of radiolabeled thymidine into uterine DNA was in fact inhibited by increasing doses of atrazine, simazine, or a common triazine metabolite DACT [18]. Other studies yielded the same result when testing for triazine effect on estrogen-stimulated uterine weight gain and progesterone receptor expression in vivo [18]. Other investigators have used estrogen-mediated

reporter systems to determine that triazines lack estrogen agonist activity [19–22]. Studies of estrogen receptor binding have discovered a very weak interaction, at only millimolar concentrations [23–26].

4. Effects on estrous cycling

The absence of direct xenoendocrine bioactivity on the part of triazines stimulated reconsideration of the test model itself, particularly of possible mechanisms related to the substantial spontaneous appearance of mammary tumors in senescence. In SD (but not F-344) female rats, control of estrous cycling declines as the animals approach mid-age, due to a failure to ovulate regularly. Estrogen secretion continues from the unovulated ovarian follicles, and vaginal cytology continues to display a cornified or keratinized epithelium. This epithelial pattern is normally visible only every fourth or fifth day of each regular cycle ('estrus'), and the repeated or continual occurrence is referred to as 'persistent estrus.'

Because the cornified vaginal cytology represents elevated estrogen secretion, a persistent estrus signals a continual high-estrogen *milieu*. This is an important development because rodent mammary tumors are so hormone-dependent. Thus, an elevated estrogen environment might be expected to correlate with mammary tumor growth in senescence. Although this prediction is reasonably well accepted, (if not heretofore directly proven), a further prediction has never been solidly established, namely that *manipulation* of estrous cycling patterns would alter mammary tumor expression in the same direction. To apply this proposal to triazine-associated mammary tumor incidence, the question was asked whether the enhanced tumor responses could correlate with, or even derive from, enhanced estrous cycle disruption in triazine-treated animals.

Results of earlier chronic studies of atrazine-fed SD female rats had suggested that treatment groups with a greater number or earlier appearance of tumors also displayed an earlier persistent estrus [2]. Not only did atrazine-treated SD rats show a higher incidence of total days in estrus and earlier mammary tumor development, but identically treated F-344 female rats were resistant to both estrous cycle disruption and mammary tumor growth [2].

Specific experiments were designed to explore this relationship more directly. Animals were treated for 24 months with atrazine and tumorigenic development was carefully monitored through necropsy. Vaginal cytology was monitored daily for 2-week intervals, alternating with two weeks without sampling. Although the in-life and necropsy portions of the studies are complete, estrous cycle analyses are incomplete as this document is being prepared. We report here lifetime tumor responses in SD female rats fed atrazine, and these results are related to estrous cycling profiles in these same animals, during the first 38 weeks on test.

Fig. 4 demonstrates that estrous cycle disruption normally appears at an early age in SD rats, independent of treatment, and that continuous feeding with atrazine at 400 ppm enhanced the rate of disruption compared to lower dosing levels and controls. Normally cycling animals should display estrus about 20–25% of all days (once every 4–5 days). In the present study, significant disruption began as early as 13–14 weeks on test, even among controls, who were only 5–6 months old at this time. The percentages of days in estrus rose to exceed 70% (i.e. more than 7 of every 10 days with a high internal estrogenic environment). Animals fed 400 ppm displayed an even greater development of increased estrous days than the other dose groups. Note the compelling similarity of the plots in Fig. 4 with the mammary tumor incidence plots of SD rats in Fig. 2.

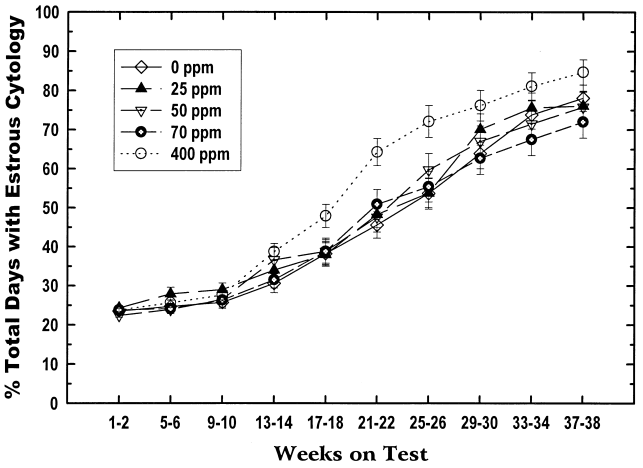

Fig. 4. Percent of total days with estrous cytology of SD female rats fed atrazine. Animals were fed atrazine in rat chow ad libitum and vaginal cytology was monitored daily for 2-week intervals, alternating with 2 weeks' rest. Symbols represent mean ± S.E. of 60 rats/treatment. There were significant effects of both dose and time ($P < 0.05$, 2-way ANOVA). Mean values of the 400 ppm group were significantly higher than 0 ppm control at all intervals beginning at weeks 13–14 ($P < 0.05$). From weeks 17–18 onward, all group means were significantly higher than their initial mean at 1–2 weeks ($P < 0.05$).

5. Mammary tumor incidence correlates with estrous cycling

A number of correlative analyses were conducted among the various parameters and a simple, yet instructive, relationship is illustrated in Fig. 5. For animals who developed mammary tumors (174/300) at any point in the 2-year atrazine feeding study (all doses combined for this figure), the percentage of total days in estrus during test weeks 1–38 was calculated for each animal, and this percentage was plotted versus the week of palpation of the first mammary tumor. Although there was a considerable variability of plotted points, the data nevertheless described a significant trend (Fig. 5).

The results suggest that animals with a very high percent

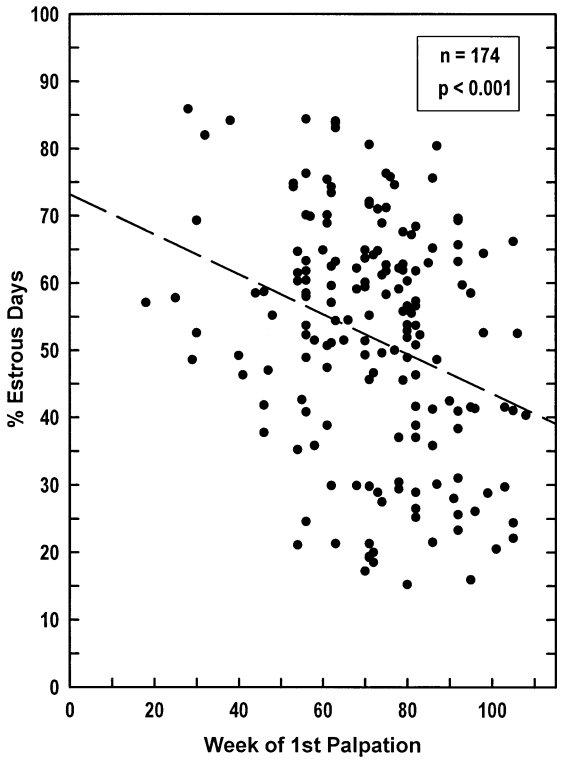

Fig. 5. Correlation between percent of total days with estrous cytology of SD female rats fed atrazine with the week of initial palpation of proven mammary tumors. Ordinate points are the summation of all days with an estrous (keratinized) vaginal cytology patten, weeks 1–38 on test, with the week of initial palpation of a tumor proven at necropsy. Graph is a composite of all doses of animals with tumors (174/300) in groups illustrated by Fig. 4, $r = 0.299$, $P < 0.001$).

estrus had a significant tendency to develop mammary tumors earlier in life, and that animals with more normal estrous cycles tended to develop mammary tumors later in life. This significant regression relationship did not always occur at each dose, due to smaller population sizes. Nevertheless, the overall effect clearly demonstrates the relationship between control of estrous cycling and mammary tumors in SD rats, and suggests that alteration of this control, by xenobiotic treatment or by natural aging, should influence the risk or timing of mammary tumor appearance.

6. Effects on the pituitary luteinizing hormone (LH) surge

In rats and other mammals, ovulation is dependent upon a single massive secretion of pituitary gonadotropins FSH and LH. In SD rats, it has been amply demonstrated that age-related ovulatory failure, that leads to episodes of persistent estrus, results from inadequate or absent LH surges [27], due primarily to deficits of neuroendocrine function responsible for generating gonadotropin surges [28]. It was decided to investigate LH surge capacity in atrazine-treated SD rats. A number of studies have now been conducted and an example of one is summarized here.

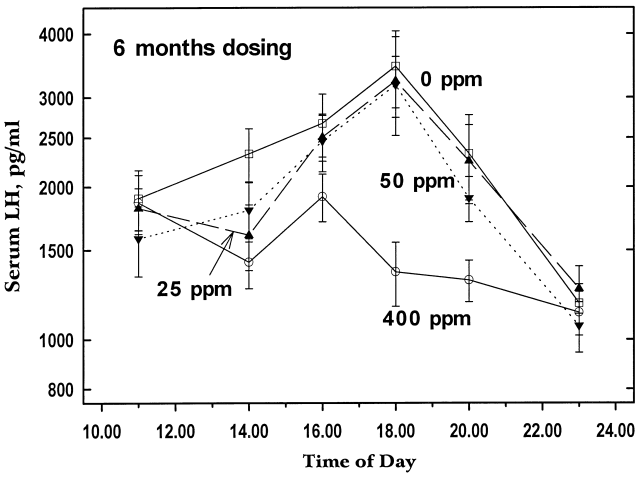

Fig. 6. Serum LH in female SD rats fed atrazine in diet for 6 months, then ovariectomized, implanted with a silastic capsule containing estrogen and sacrificed at indicated times 3 days later. Note the ordinate scale is logarithmic. Points represent means ± S.E. of 20 animals/dose. The silastic capsule contained 4 mg/ml 17-β estradiol in sesame oil and produced steady-state plasma levels of 100 pg/ml. There were significant effects of both time and dose (2-way ANOVA, $P < 0.05$). Mean values for 400 ppm at 14.00, 16.00, 18.00, and 20.00 were significantly different from control values at 0 ppm ($P < 0.05$).

Beginning at 6–8 weeks of age, groups of SD female rats were placed on either standard diet or diet containing atrazine at 25, 50 or 400 ppm. After 6 months, all the animals were ovariectomized and each was implanted with a silastic capsule containing estradiol. Three days after implantation, subgroups at each dose were sacrificed at one of several time intervals, and serum was prepared from trunk blood and analyzed for LH. Results, shown in Fig. 6, demonstrated a greatly reduced LH surge in animals fed 400 ppm atrazine, compared with groups at 25 or 50 ppm or the controls. Because other animals dosed with 400 ppm atrazine displayed an increased percentage of days with estrous vaginal smears, and later an increased incidence of mammary tumors (but none of these observations were made at 25 or 50 ppm), the LH result strongly suggests that the cause of estrous cycle disruption lies in the ability of atrazine to interfere with neuroendocrine control of ovulation. Additional studies [29] have confirmed the effect of atrazine on LH surge suppression, after acute (3 days) or subchronic (4 weeks) administration.

7. Discussion and summary

The results of our studies suggest that previously observed increases, or an earlier appearance, of mammary tumors in atrazine-treated female SD rats are a result of interaction between treatment and the process of normal aging in these test animals. Triazines have no intrinsic hormone activity and cannot support carcinogenesis on their own. The mammary tumor response is limited to one strain

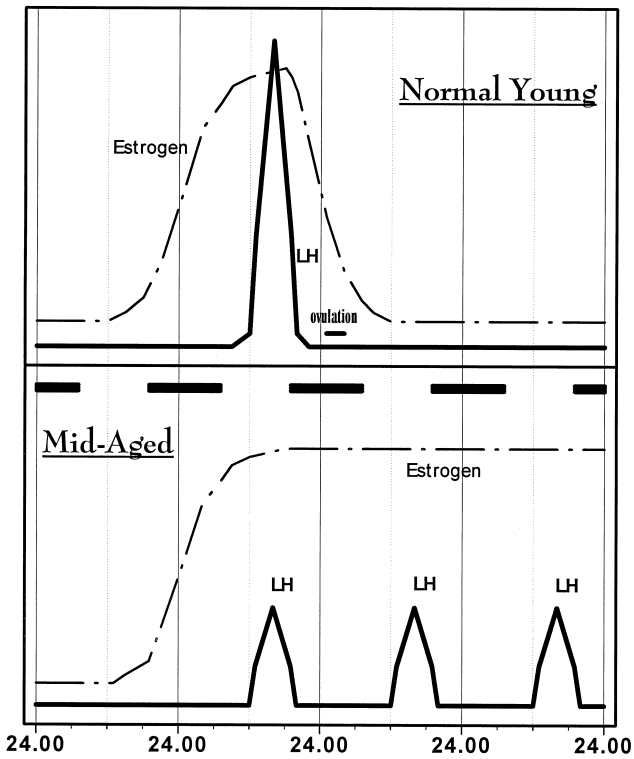

Fig. 7. Schematic illustration that compares LH Surges in young and mid-aged SD female rats. In young animals, rising estrogen from ovarian follicles triggers a massive LH surge once every 4th or 5th day. In mid-age, neuroendocrine deficits result in an LH surge insufficient for ovulation. Thus, estrogen secretion is maintained from unovulated follicles, as suboptimal surges are repeated.

of one species, in females that have an already high spontaneous tumor incidence. Further, the tendency of the Sprague-Dawley strain to lose control of LH surges and ovulation, and to enter high-estrogen episodes of persistent estrus, has been shown to be exacerbated by atrazine dosing, but only at levels previously associated with the mammary tumor responses. A different albino rat strain, Fischer-344, responds neither to aging nor to atrazine treatment in regard to mammary tumor development or persistent estrous episodes. Thus, it appears that, in the chosen SD rat test model, atrazine exacerbates an already great tendency for age-related pathology.

Fig. 7 is a graphic illustration of this effect. As SD rats age, LH surges fall below a threshold necessary to stimulate ovulation. As a result, estrogen secretion continues from unovulated ovarian follicles. It is proposed that triazine dosing at very high levels accomplishes the same result, at a somewhat earlier age, producing a earlier environment of elevated endogenous estrogen, to support earlier spontaneous mammary tumor growth.

It is highly unlikely this response in SD rats bears any relevance to humans. Menopause in women is more widely believed to result from exhaustion of primordial ovarian follicles, and estrogen levels decline in women during the perimenopausal phases [30]. Furthermore, the requisite treatment levels of atrazine needed to achieve the observations in rats of LH suppression, estrous cycle disruption or enhanced mammary tumor responses are far above any reasonable exposure level. Treatment levels not far below 400 ppm (50–70 ppm) have been shown to have no effect on estrous cycling or mammary tumor incidence. The current permitted average maximum contaminant level (MCL) of atrazine in ground water is 3 parts per billion, or more than 20 000 times lower than 70 ppm. Therefore, from a safety margin standpoint, and because the estrous cycling effect is rather unique to the SD rat strain and not applicable to humans, it is very reasonable to conclude that current exposure levels to atrazine present no threat to human health in this regard.

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