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INDIRECT INFLUENCE OF s-TRIAZINES ON RAT GONADOTROPIC MECHANISM AT EARLY POSTNATAL PERIOD

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Summary—Daily s.c. injections of atrazine and deethylatrazine to rat mothers during pregnancy only or during pregnancy and lactation influenced the pituitary–gonadal axis of male and female offsprings. In female and male offspring, slow maturation of gonadotropic system is evident and as a consequence modified male and female pituitary 5α-R activity is present. The number of specific steroid-hormone receptor sites at the offsprings' gonads is unchanged if the mothers were treated only during pregnancy, but 5α-DHT prostrate receptors are strongly inhibited in offspring of mothers treated with atrazine and deethylatrazine during pregnancy and lactation.

INTRODUCTION

The use of pesticides is still rising continuously. There is growing evidence that certain pesticides can adversely affect the hormonal balance in mammals. Most toxic substances entering the circulation can cross the placenta, the brain barrier and result in various risks to the conceptus [1]. Many extensive studies have been conducted on organochlorine pesticides and their action on the reproductive system [2] and it was found that some of them produce changes in hormone-dependent organs [3, 4]. The question of whether long-term exposure to minimal levels of pesticides will affect the future generations is still open. It is known that a number of pesticides or their biodegradation products suggest a mutagenic and carcinogenic risk [5]. Some herbicidal precursors, such as carbamates, and triazines, nitrosoamines and nitroso-derivatives, possess the same characteristics [5, 6]. s-Triazine herbicides also exert a certain inhibitory effect on hormone-dependent reaction at the rat ventral prostrate [7, 8], hypothalamus [7] and anterior pituitary [9]. Very dynamic changes of pituitary 5α-reductase activity at early postnatal period of male and female rats [10] was the aim of the present study, in order to determine the possible indirect influence of an s-triazine herbicide (atrazine)§ and its biodegradation product (deethylatrazine) on pituitary testosterone metabolism, DHT and estradiol–receptor complex formation in young male and female offspring of rat mothers treated with herbicides either during pregnancy only or during pregnancy and lactation.

§Abbreviations: Atrazine, 2-chloro-4-ethylamino-6-isopropylamino-s-triazine; deethylatrazine, 2-chloro-4-amino-6-isopropylamino-s-triazine; testosterone, 17β-hydroxyandrost-4-ene-3-one; 5α-dihydrotestosterone (5α-DHT), 5α-androstan-17β-ol-3-one; 3α-diol, 5α-androstane-3α, 17β-diol; androstenedione, androst-4-ene-3,17-dione; 5α-androstanedione, 5α-androstane-3,17-dione; 5α-reductase (5α-R), 3-oxo-steroid 4-ene-dehydrogenaseC(1.3.99.5); 3α-hydroxysteroid dehydrogenase (3α-HSD) (EC 1.1.1.50); 17β-hydroxysteroid dehydrogenase (17β-HSD) (EC 1.1.1.63).

MATERIAL AND METHODS

Chemicals

[4-^{14}C]Testosterone (sp. act. 2.15 GBq/mmol) was obtained from the Radiochemical Centre, Amersham, Bucks, U.K. and purified by TLC a few days beofre use. [1, 2, 3, 4, 5, 6-^{3}H]Dihydrotestosterone (sp. act. 4.99 TBq/mmol) and [2, 4, 6, 7, 16, 17-^{3}H]estradiol (sp. act. 4.81 TBq/mmol) were obtained from New England Nuclear and used without further purification. Unlabelled testosterone, 5α-dihydrotestosterone, 5α-androstane-3α$_T$ 17β-diol, androst-5-ene-3,17-dione and 5α-androstane-3,17-dione were obtained from Steraloids Inc., Pawling, NY, U.S.A.

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine) and deethylatrazine (2-chloro-4-amino-6-isopropylamino-s-triazine) were obtained from Radonja, Sisak, Yugoslavia, and were purified by recrystallization and TLC before use.

All other chemicals were commerical preparations and analytical grade.

The buffers used were: glucose Krebs–Ringer solution, pH 7.4, TED (Tris 0.01 mol/l, EDTA 0.001 mol/l, dithiothreitol 0.001 mol/l; adjusted to pH 7.4) and TEDG (Tris 0.05 mol/l, EDTA 0.0015 mol/l, dithiothreitol 0.0015 mol/l; adjusted to pH 7.4, containing 20% glycerol).

Dextran-coated charcoal (DCC) suspension contained activated charcoal Norit A (0.5%, w/v) and dextran T$_{60}$ (0.05%, w/v) in TED or TEDG buffer.

Animals, treatment and tissue

Fisher strain female rats and their offspring were caged with food and water *ad libitum* on a lighting schedule of 12 h light: 12 h dark.

Female rats (~180 g) from the first day of pregnancy were treated by s.c. injections of atrazine or deethylatrazine (1.66 mg/100 g body wt daily). Solutions were prepared fresh daily and all s.c. injections were made in 0.1 ml paraffin oil/animal. Female rats were treated during pregnancy only or during pregnancy and lactation. Young rats up to 28 days of age fed from their own mothers.

Body weights of the treated and control mothers as well as their offspring were followed weekly and there were no significant differences between the control and different treated groups.

Male and female offspring were sacrified by decapitation on 21st or 28th day of life. The anterior pituitaries were removed immediatly and blood traces washed out in glucose Krebs–Ringer solution before incubation. The ventral prostate or uterus tissues were collected from each group for cytosol preparation.

Pituitary enzymatic activities

Male or female immature rat pituitary (one or more depending to the weight) was immersed in 2 ml of glucose Krebs–Ringer solution, pH 7.4, containing 1.332 kBq [4-^{14}C]testosterone (about 0.59 nmol). Incubation procedure, isolation, identification and quantification of testosterone metabolites were performed according the method previously described [11].

Determination of cytosol estradiol receptors

The immature rat uteri dissected free of blood traces or adhering fat, were homogenized by Ultraturrax homogenisator (3 × 15 s time period) in TED buffer by holding the whole system in an ice bath. The homogenate was centrifuged at 105,000 **g** for 60 min at 4°C. The supernatant was used as cytosol for determination of estradiol receptors. Protein concentration in the cytosol was in the range 0.7–1.3 mg/ml.

Estrogen receptor activity was determined after incubation of 200 μl of cytosol with 25 μl [2, 4, 6, 7, 16, 17-^{3}H]estradiol (0.13–1.3 nM) for 20 h at 4°C. Parallel incubations contained unlabelled diethylsilbestrol (DES 0.26 μM) as competitor. Bound ligand was determined by exposing the incubation mixture to 500 μl of DCC solution, in order to strip unbound steroid [12]. The mixture was shaked vigorously for 30 min at 4°C, and then centrifuged at 2200 g for 30 min. A portion (500 μl) of supernatant fraction was added to tritium scintillator (10 ml) and counted for radioactivity.

Determination of cytosol DHT receptors

The immature rat prostates were homogenized (holding the whole system in an ice bath) in TEDG buffer by a glass–Teflon tissue homogenizer (Kontes Glass Co., NJ). The homogenate was centrifuged at 105,000 **g** for 60 min at 4°C. The supernatant fraction with the protein concentration from 1.0 to 1.5 mg/ml was used for further determination of DHT receptors.

Cytosol (200 μl) was incubated with 25 μl [1, 2, 3, 4, 5, 6-^{3}H]dihydrotestosterone (concentration from 1 to 9 nM) for 20 h at 4°C. Parallel incubations contained unlabeled DHT (0.2 μM) as competitor.

Continuation of the procedure is the same as described before for determination of estradiol receptors.

RESULTS

Daily s.c. injections of atrazine and deethylatrazine to rat mothers during pregnancy did not induce any significant changes at pituitary 5α-R, 3α-HSD or 17β-HSD activities of 28-day-old male offspring (Table 1). Female 28-day-old offspring possess significantly higher activity of pituitary 5α-R (Table 2) than males (1609 vs 623 pg/mg for control–intact and 1614 vs 736 pg/mg for control–oil). Treatment during pregnancy with atrazine induces further increase of 5α-R activity (2127 vs 1614 pg/mg). 3α-HSD acts as a second step in the testosterone metabolism and results obtained after treatment of mothers with atrazine or deethylatrazine show (Table 2) a tendency of increasing activities (1195 vs 748 and 1027 vs 748). This is the single evidence that treatment of mothers during pregnancy induces, through the placental barrier, long-term effects on female offspring.

Long-term treatment of rat mothers during pregnancy and lactation with atrazine and its metabolite deethylatrazine induces significant decrease in the formation of pituitary 3α-diol (394 and 486 vs 678 pg/mg) in 21-day-old male offspring (Table 3). 5α-DHT formation is significantly decreased in atrazine group (659 vs 870 pg/mg) in 21-day-old male offspring. Androstenedione and androstanedione formation have not been changed after treatment either with atrazine or deethylatrazine. While there are no differences in the weights of pituitaries (2.7 vs 3.2 mg), decrease of the enzymatic activities is a result of treatment of mothers during pregnancy and lactation and direct transfer of pesticide through the mother's milk. From Table 3 it is evident that male pituitary 3α-diol formation is more reduced after application of atrazine (42%) than with deethylatrazine (29%), respectively. 5α-DHT formation after atrazine treatment is reduced by 25% and after deethylatrazine no significant difference has been found (Table 3).

In spite of the 5α-R activity being higher in pituitary 21-day female controls (Table 4) than in the same aged males (5387 vs 870 pg/mg), female enzymatic systems, no matter which (Table 4) are

Table 1. Conversion of [4-^{14}C]testosterone to the metabolites in the anterior pituitary of male rats aged 28 days from mothers treated with atrazine and deethylatrazine during pregnancy

Treatment	Tissue wet wt (mg)	pg metabolite/mg wet tissue			
		5α-Androstane-3α,17β-diol	5α-Dihydro testosterone	Androst-4-ene-3,17-dione	5α-Androstane-3,17-dione
Control (intact)	4.0 ± 0.20 (5)	240 ± 28.8	623 ± 38.2	577 ± 28.8	190 ± 25.2
Control (oil)	3.6 ± 0.39 (5)	322 ± 32.4	736 ± 51.5	729 ± 83.0	209 ± 18.5
Atrazine 1.66 mg/ 100 g body wt per day	3.7 ± 0.27 (5)	291 ± 21.6	728 ± 61.3	789 ± 138.8	195 ± 20.2
Deethylatrazine 1.66 mg/ 100 g body wt per day	3.9 ± 0.27 (5)	310 ± 50.5	656 ± 67.8	659 ± 46.4	180 ± 8.7

Values are mean ± SE; ( )—number of samples. Statistical evaluation was done with the Student's *t*-test.

Table 2. Conversion of [4-^{14}C]testosterone to the metabolites in the anterior pituitary of female rats aged 28 days from mothers treated with atrazine and deethylatrazine during pregnancy

Treatment	Tissue wet wt (mg)	pg metabolite/mg wet tissue			
		5α-Androstane-3α,17β-diol	5α-Dihydro testosterone	Androst-4-ene-3,17-dione	5α-Androstane-3,17-dione
Control (intact)	2.4 ± 0.13 (6)	902 ± 74.5	1609 ± 168.4	541 ± 40.4	543 ± 57.0
Control (oil)	2.7 ± 0.22 (5)	748 ± 77.7	1614 ± 50.0	488 ± 40.4	394 ± 29.3
Atrazine 1.66 mg/ 100 g body wt per day	2.6 ± 0.10 (5)	1195 ± 152.0	2127 ± 186.9 $P < 0.05$	558 ± 63.0	435 ± 38.5
Deethylatrazine 1.66 mg/ 100 g body wt per day	2.5 ± 0.14 (4)	1027 ± 303.9	1689 ± 92.8	486 ± 29.1	478 ± 64.2

Values are mean ± SE; ( )—number of samples. Statistical evaluation was done with the Student's *t*-test; *P*—significant difference compared to control (oil).

Table 3. Conversion of [4-^{14}C]testosterone to the metabolites in the anterior pituitary of male rats aged 21 days from mothers treated with atrazine and deethylatrazine during pregnancy and lactation

Treatment	Tissue wet wt (mg)	pg metabolite/mg wet tissue			
		5α-Androstane-3α,17β-diol	5α-Dihydro-testosterone	Androst-4-ene-3,17-dione	5α-Androstane-3,17-dione
Control (intact)	2.19 ± 0.23 (6)	873 ± 122.4	907 ± 48.3	519 ± 27.4	443 ± 43.0
Control (oil)	2.7 ± 0.16 (5)	678 ± 56.7	870 ± 59.9	454 ± 29.1	461 ± 72.1
Atrazine 1.66 mg/ 100 g body wt per day	3.2 ± 0.24 (6)	394 ± 44.9 $P < 0.01$	659 ± 74.0 $P < 0.05$	570 ± 89.2	272 ± 60.3
Deethylatrazine 1.66 mg/ 100 g body wt per day	2.6 ± 0.15 (5)	486 ± 45.7 $P < 0.05$	803 ± 37.2	447 ± 35.1	418 ± 31.2

Values are mean ± SE; ( )—number of samples. Statistical evaluation was done with the Student's *t*-test; *P*—significant difference compared to control (oil).

Table 4. Conversion of [4-^{14}C]testosterone to the metabolites in the anterior pituitary of female rats aged 21 days from mothers treated with atrazine and deethylatrazine during pregnancy and lactation

Treatment	Tissue wet wt (mg)	pg metabolite/mg wet tissue			
		5α-Androstane-3α,17β-diol	5α-Dihydro testosterone	Androst-4-ene-3,17-dione	5α-Androstane-3,17-dione
Control (intact)	3.0 ± 0.36 (5)	1872 ± 168.0	6432 ± 855.9	758 ± 69.7	760 ± 96.9
Control (oil)	2.7 ± 0.36 (5)	1879 ± 148.2	5387 ± 511.1	883 ± 107.8	736 ± 109.0
Atrazine 1.66 mg/ 100 g body wt per day	2.3 ± 0.36 (5)	2242 ± 294.5	5539 ± 410.5	775 ± 73.8	745 ± 108.2
Deethylatrazine 1.66 mg/ 100 g body wt per day	3.6 ± 0.14 (5)	2079 ± 209.0	4558 ± 336.8	606 ± 37.5 $P < 0.05$	543 ± 48.1

Values are mean ± SE; ( )—number of samples. Statistical evaluation was done with the Student's *t*-test; *P*—significant difference compared to control (oil).

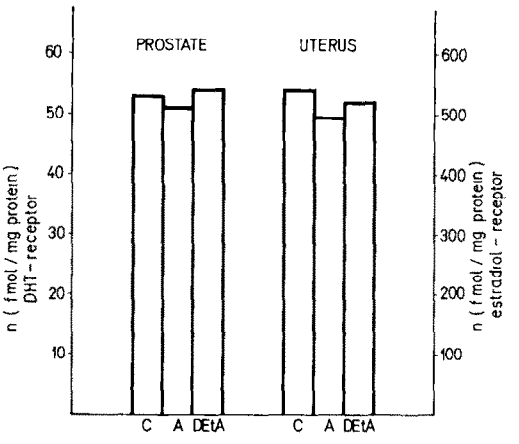

Fig. 1. Number of specific binding sites (n) for DTH-receptor in prostate and estradiol-receptor in uterus of 28-day offspring from control (C) mothers and s.c. treated (1.66 mg/100 g body wt per day) with atrazine (A) or deethylatrazine (DETA) during pregnancy.

rather stable and no change is evident in the hormone responsible enzymatic systems. The significant difference in pituitary 21-day female androstenedione formation after treatment with deethylatrazine has to be explained in further experiments.

Figure 1 summarizes the results of hormone-receptor specific binding sites' determination in gonads of 28-day-old offspring. Treatment of mothers with atrazine and deethylatrazine during pregnancy did not change the number of specific binding sites for prostate DHT-receptor (53 vs 51 vs 54 fmol/mg protein) or for uterus estradiol-receptor (540 vs 495 vs 520 fmol/mg protein). Prolonged treatment of mothers during lactation with the same concentrations of atrazine and deethylatrazine induces a significant reduction in number of specific binding sites for prostate DHT-receptor of 21-day offspring (Fig. 2). Scatchard analysis [13] gives the number of specific binding sites for prostate DHT-receptor for the control group as 32 fmol/mg, for the atrazine group

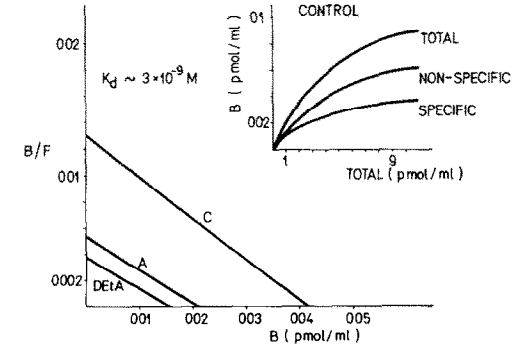

Fig. 2. Scatchard analysis of the data for prostate DHT-receptor complex formation of 21-day offspring from control (C) mothers and s.c. treated (1.66 mg/100 g body wt per/day) with atrazine (A) or deethylatrazine (DEtA) during pregnancy and lactation.

25 fmol/mg and for the deethylatrazyine group 19 fmol/mg protein, respectively.

DISCUSSION

Previous studies in our laboratory have shown that atrazine and prometrine, as s-triazines compounds, possess direct influence on testosterone metabolism in the pituitary of male rats [9]. In the present study, using an indirect system to perform the influence of atrazine or its metabolite, deethylatrazine, female rats were treated during pregnancy or during pregnancy and lactation. The herbicide influence was checked in male and female 21-day and 28-day offspring, for testosterone metabolism in the pituitary and for DHT-receptor and estradiol-receptor formation in prostate and uterus.

Ability of the hormone-receptor complex formation is a prerequisite for hormone activity. The 28th day of life is selected because at that time strong sexual differentiation between male and female offspring is present.

Determination of the number of specific binding sites have been performed in the prostate and uterus tissues. The specific binding has been achieved by measuring total and non-specific binding and the number of specific binding sites has been evaluated using Scatchard analysis [13].

While our previous investigations do not give any results about the number of specific binding sites on specific receptors for DHT and estradiol, under the influence of herbicides, it is not possible to compare the present results for the number (n) of DHT binding sites at prostate and estradiol binding sites of the uterus of 28-day-old rats; we can conclude that after treatment of mothers during pregnancy with atrazine or deethylatrazine, n remains unchanged, for DHT binding in prostate is around 50 and for estradiol binding in uterus is over 500 fmol/mg protein (Fig. 1).

Treatment of females during pregnancy and lactation indicates reduction of n in prostate DHT receptors of 21-day offspring, in the direction control (oil) > treated mothers with atrazine > treated mother with deethylatrazine (32 vs 25 vs 19 fmol/mg protein). The stronger reduction of n after treatment with deethylatrazine than with atrazine has to be explained.

Determination of pituitary 5α-R, 17β-HSD and 3α-HSD activities in 28-day male offspring have shown that if mothers are exposed to 1.66 mg/100 g body wt of atrazine daily during pregnancy, it does not induce any significant change in these enzymatic activities of offspring (Table 1). Previous results [9] have shown that after *in vitro* or *in vivo* treatment of adult male rats with atrazine, strong inhibition of pituitary 5α-R and 3α-HSD has been found. The present results with 28-day offspring (Tables 1 and 2) could be explained by long-term influence of herbicides on the enzymatic systems, inducing inhibition

in development. The reduction is more evident through the higher pituitary 5α-R at females [10] than in males, where the enzymatic systems at that age are more stable. If the treatment of mothers has been continued after pregnancy during lactation (Table 3), inhibitory effect on pituitary enzymatic systems of 21-day male offspring is present, while the herbicide enters the young organism during feeding with milk.

Female 28-day offspring whose mothers have been treated with atrazine or deethylatrazine during pregnancy possess the increased 5α-R and 3α-HSD activities in the pituitary vs control (Table 2). Previous published results [10] have shown that there is a significant increase of pituitary 5α-R activity in females from 0 to 14 days of life and at that time is almost 9 times higher than in males, with a later decrease which is, on day 50 of life, only 30% of that in males.

Significantly higher pituitary 5α-R activity in 28-day-old females from mothers treated with atrazine during pregnancy (Table 2), than from the control (oil) or control (intact) mothers, is probably the reason for slower maturation of gonadotropic mechanism, in spite of which body weight did not show any difference between the groups. The tendency of increase after treatment with deethylatrazine, which has rather weak herbicidal activity, has given results with some inhibition effect on the development of gonadotropic system but not as much as with atrazine.

Table 4 presents results of pituitary 5α-R, 17β-HSD and 3α-HSD activities in 21-day female offspring from mothers treated during pregnancy and lactation with atrazine or deethylatrazine. 5α-R and 3α-HSD activities do not have any significant difference from controls, and it means that inhibition effect of herbicide from the lactating mothers is compensated by the higher activity of 5α-R corresponding to 5α-R activity of 19–20-day-old female rats [10].

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