In-ovo Exposure to 2,3,7,8-TCDD Results in Delayed Reproductive Maturation in the Domestic Chicken.

K. R. Alonso[1], M. M. Peden[1], D. Henshel[2], and R. L. Dickerson[1]
[1]Department of Environmental Toxicology, Clemson University, PO Box 709, Pendelton, SC 29670
[2]School of Public and Environmental Affairs, Indiana University, Bloomington, IN.

1. Introduction

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) a by-product of paper and chemical manufacturing and incineration is considered the most toxic of dioxins. TCDD causes a variety of toxic responses, including wasting syndrome, carcinogenicity, dermal toxicity, endocrine alteration, immunotoxicity, porphyria, thymic atrophy, and reproductive toxicity. This compound is a well known anti-estrogen which binds with high affinity to the Ah receptor[1]. TCDD has been reported to have reproductive and teratogenic effects in several strains of mice, rats, rabbits, and chickens[2]. Reproductive effects have been shown in the F_1 and F_2 generations of rats which received a dose of 0.01 mg/kg/day while no effects were noted in the P_0 generation[3]. Male rats exposed gestationally showed effects in spermatogenesis, daily sperm production, and adult sexual behavior[3]. Male rat reproductive systems exposed in utero appear to be twice as sensitive to 2,3,7,8-TCDD as when exposed in adulthood[3]. Air sack injections of 0.1 ug/kg 2,3,7,8-TCDD on embryonic day 4 in domestic chickens caused significant decreases in 17b estradiol in four day old hatchlings ($p<0.05$)[4]. This project was designed to study the reproductive developmental, and immunotoxic effects of TCDD on wildlife at the Times Beach, Missouri superfund sight. Oil contaminated with TCDD which was sprayed along the roads as a dust measure prompted a governmental buyout of the area. White Leghorn chickens were used as surrogate species for the resident wild turkeys on sight. TCDD was injected into the chickens at levels which matched the on sight levels of the contaminated and remediated soil which were 200 and 1 ppb respectively. The offspring were then followed through development to determine any alterations in reproductive maturity through the use of a four way breeding study. F_1 low adult females began egg production approximately two weeks later than did F_1 control females. By week eight the production of eggs was equivalent. Semen quality was measured in both F_1 low adult males and F_1 control adult males. Both volume and sperm number were reduced in the F_1 low male as compared to the F_1 controls. However, over 90% fertility was observed in all four pairing groups indicating that the reduction in semen quality was insufficient to alter reproductive success. A special thanks to USFWS & NIEHS ES04696 for funding this research.

2. Methods

Pretreatment

The animals were acclimated to their surroundings for four weeks before treatment began. During these four weeks eggs were collected twice daily. At the end of the acclimation time the adult birds were bled. This blood was used for endocrine function analysis in order to provide a base line for comparison after treatment.

Treatment

The treatment phase lasted six weeks. The birds were injected i.m. in the thigh twice weekly

and weights taken once a week. The control group received .25 ml of olive oil. The remediated model group received .25 ml of a .12 ug/ml solution of TCDD in olive oil, and the contaminated model group received .25 ml of a 24 ug/ml solution of TCDD in olive oil. The eggs were collected twice daily and set once a week. The eggs were candled for viable embryos on day 5, 9, 14 and 18. On day 18 they were transferred to the hatcher. The eggs hatched on days 21-22. Hatchling weights were taken on the day of hatch. In each hatch five chicks from each treatment group were bled and sacrificed on day 14. At the end of treatment all adults, except one group of high dose birds, were bled and sacrificed. The blood was used for endocrine function analysis. After a month the remaining group of high dose birds were sacrificed. From the last hatch of chicks four males and four females, from the control and low dose groups were allowed to reach sexual maturity. Onset of egg production and egg viability were measured. At age 30 weeks the F_1 generation was sacrificed and gonadal weights, blood, and eggshell glands were taken.

Analysis

Hormone Level Determinations (Radio-immunoassay) - To extract hormones from plasma modified ICN methods were used from testosterone and 17b-estradiol radioimmuno-assay kits (ICN). Hormone extractions required mixing and separation of plasma in a 3:2 ethyl acetate:hexane solution (solvent A). Solvent A, containing the hormones was transferred to glass scintillation vials and evaporated with compressed air. Samples were reconstituted with steroid diluent and incubated for 30 min at 37°C to resuspend the steroid hormones. The assay utilized 500ul reconstituted sample mixed with 100ul anti-hormone. To this 100ul tritiated (H^3) hormone was added and the resulting mixture incubated at 2-8°C for 1 or 2 hours for 17b-estradiol and testosterone, respectively. Two hundred microliters of a charcoal dextran solution was added, and the solution shaken and incubated for 20 min at 2-8°C to remove any unbound steroid hormone. Samples were centrifuged in a Beckman GPR centrifuge for 15 min at 2500 rpm to remove charcoal. Supernatant was added to 5 ml Ecoscint (National Diagnostic) and counted for 2 min in a Beckman LS 1701 scintillation counter. Results were compared to standard curves to determine the plasma concentration of 17b-estradiol and testosterone.

Hormone Receptor Assay - Eggshell gland was homogenized using liquid nitrogen and a mortar and pestle followed by homogenization with a power driven glass homogenizer in TEDG buffer (50 mM Tric-HCl, 1 mM EDTA, 10% glycerol, 10 mM sodium molybdate, and 50 mM dithiothreitol pH 7.5). The homogenate was centrifuged in a Beckman GPR at 2500 rpm to separate the cytoplasmic from the nuclear fraction. The supernatant of this portion was run through a Beckman L8-M ultracentrifuge at 33800 rpm to separate the microsomal portion. This assay was run on both cytosolic and nuclear fractions. The assay was performed in a 96 well plate with a U shaped bottom. Sample (50 ul) was added to each of six wells. To three of the wells H^3 hormone (2 ul) was added to the other three wells H^3 hormone + 100x excess of competitor was added and allowed to incubate for 30 min at 30°C. Afterward 100 ul (1.5625% charcoal, .15625% dextran solution) was added and mixed at 4°C for 15 min on a rotating plate. The plate was centrifuged for 15 min at 2500 rpm to remove charcoal. Supernatant (50 ul) was added to scintillation fluid (3 ml) and counted for 2 min in a Beckman LS 6500 liquid scintillation counter. The difference between the competitive binding and tritiated binding determined the amount of receptors. Protein concentration determination of the samples was determined by the Bradford protein assay[5].

3. Results

The effects of *in-ovo* exposure to 2,3,7,8-TCDD on F_1 generation egg production can be seen in Table 1. The table shows egg production of low and control dose hens regardless of the roosters with which they were associated. At week eight, egg production of the two groups normalized. Week nine egg production displays the first week the roosters were moved to cages containing hens of the opposite dose group. No obvious changes were noted. Overall fertility seemed to be lower in

F_1 low dose hens this was statistically significant at week 2 as can be seen in Table 1. Remembering that week nine values represent hens with roosters of the opposite dose group, no fertility alterations were observed.

Sperm quality was measured at 23 weeks of age (Table 2). Collection of sperm was limited to only two controls and one low dose rooster. Volume, amount, and viability of sperm from the controls was considered to be normal, while the volume from the low dose was considered below normal[6]. Motility of sperm was normal for the controls and limited in the low dose. Percent of abnormal sperm in the controls was low ; however, a high percentage of abnormal sperm was observed in the low dose[6]. All sperm data was qualitative and not quantitative therefore no statistics were run.

Physiological alterations were assessed by total body weights and percent gonad weight to body weight at the end of the study (Table 3). Female body weights were lower in both groups than those of males. Control had lower body weights than low by sex. Low dose male body weight were statistically different from control males ($p< 0.05$). Percent gonad weight to body weight in low dose birds increased over controls in both sexes; however, this was not statistically significant. Comb length of these birds was unaffected by *in-ovo* exposure.

F_1 generation hormone levels were determined by radio-immunoassays. Table 4 shows estradiol levels for both sexes were decreased in the low dose; however, neither was significantly different from control. Plasma testosterone levels were lowered in hens and raised in roosters but not significantly. Estrogen receptor levels in cytosolic and nuclear fractions were lowered in the low dose as compared to controls, although not significantly. Progesterone receptor levels showed these same trends but were also not significant (Table 5).

Table 1. F_1 generation egg production and fertility

Week	Control Egg Production	Low Egg Production	Control Egg Fertility	Low Egg Fertility
1	1	0	0	0
2	8	1	62.5	0 *
3	19	9 *	84.2	100 *
4	19	13	100	84.6
5	23	18	91.3	88.9
6	25	17	100	88.2
7	27	18	100	94.4
8	23	23	100	91.3
9	24	24	91.7	87.5
10	25	23	100	95.7
11	21	24	100	95.8

* significant from control at $p<0.05$, n=4

Table 2. F_1 generation sperm quality

PARAMETERS	TREATMENT	
	Control	Low
# of Birds Collected	2	1
Volume	normal	low
Amount of Sperm	normal	low
Viability	normal	low
Motility	normal	limited
Amount Abnormal	low	high

Birds 23 weeks old.
Table 3. F_1 generation weights

	Total Body Weight (g)	Total Gonad Weight (g)	% gonad weight of body weight
Control Hen	1546.53 +/- 37.27	13.39 +/- 1.18	0.87 +/- 0.087
Low Hen	1634.35 +/- 30.87	17.92 +/- 2.25	1.09 +/- 0.13
Control Rooster	1895.43 +/- 83.86	28.25 +/- 1.22	1.5 +/- 0.10
Low Rooster	2188.95 +/- 49.64 *	35.97 +/- 4.5	1.64 +/- 0.19

*Significant from control at $p<0.05$, n=4

Table 4. F_1 Generation Plasma Hormone Levels measured at 30 weeks of age.

	Plasma Estradiol pg/ml		Plasma Testosterone pg/ml	
Hen Control	353.13 +/- 70.36	(4)	328.13 +/- 58.22	(3)
Hen Low	286.10 +/- 56.97	(4)	181.40 +/- 95.68	(4)
Rooster Control	32.39 +/- 0.89	(2)	2321.54 +/- 846.93	(4)
Rooster Low	22.86 +/- 2.86	(2)	2897.29 +/- 2522.84	(3)

($p<0.05$)

Table 5. F_1 Generation Hen Eggshell Gland Receptor Levels

	Cytosolic Estrogen Receptor fmole receptor /mg protein	Nuclear Estrogen Receptor fmole receptor /mg protein	Cytosolic Progesterone Receptor fmole receptor /mg protein	Nuclear Progesterone Receptor fmole receptor /mg protein
Control	9.089 +/- 6.77	27.6 +/- 9.66	3469.4 +/- 727.52	850.54 +/- 264.31
Low	4.48 +/- 5.01	24.97 +/- 7.7	1746.43 +/- 707.66	459.59 +/- 232.88

($p<0.05$), n=4

4. Conclusions

In-ovo exposure to a parental averge dose of 0.198ug/kg 2,3,7,8-TCDD (0.3 ug twice a wek for six weeks) results in delayed egg production and decreased fertility. Although serum steriod hormone levels were not significantly different between treatments, alterations were noted which could effect the onset of maturity and subsequent egg production as well as fertility. The decrease in

both estrogen and progesterone receptor levels in low dose hens could also attribute to the delayed onset of maturity.

5. References

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