2,3,7,8-TCDD Effects on Immune Function and Mixed Function Oxidase Activity in the Domestic Chicken: Egg Injection and *In-Ovo* Studies.

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1. Introduction

In the early 1970's, 2,3,7,8-tertachlorodibenzo-p-dioxin (TCDD) was produced as a byproduct in the manufacturing of hexachlorophene in a plant in eastern Missouri. Waste oils containing this byproduct were sprayed in the area on roadsides, dirt lots, and horse arenas as a dust control measure. This practice resulted in both isolated and widespread areas of TCDD soil contamination. Thirty-eight sites have been identified as widespread and a possible 200 more are isolated sites[1]. Times Beach, Missouri has been identified as one of the worst areas of contamination with levels as high as 1200 ppb[2]. Although contaminated areas averaged 200 ppb[3] much of the site was not contaminated. The contamination was limited to roads and roadsides; therefore, the overall site (500˜ acres) TCDD level before remediation was approximately 40ppb[4]. In 1982, the town was evacuated and designated a superfund site under CERCLA. In 1989, remediation began utilizing both covering and incineration to reach a level of 1 ppb in the contaminated areas. Currently a large herd of deer and flocks of wild turkey reside on the site. Therefore, concern has been raised as to the health of these animal popualtions. This study consisted of two phases. In the first phases domestic chicken eggs were injected with various concentrations of 2,3,7,8-TCDD and in the second phase adults were injected with dose levels which modeled the worst case scenarios of animals feeding only on the roads and rodsides of the site before and after remediation. In both of these phases immune and mixed function oxidase biomarkers were assessed.

2. METHODS

Egg Injection and Incubation:

Three hundred white leghorn eggs were randomly sorted into six treatment groups (50 eggs per group). The eggs were labeled as to treatment group and number, weighed to the nearest 0.01 gram, and washed with Betadine™. The average weight of the eggs was 60 ± 1 gram and this was used to fix the injection volume. Each egg was candled to locate the air sac and its boundary was marked on the egg shell in pencil. The egg tops were wiped with 70% ethanol and a Dremel™ tool equipped with a 1/16˜ burr was used to perforate the shell inside of the air sac line. A sterile Hamilton syringe was used to inject six ul of either 0, 20 pg/ml, 200 pg/ml, 2 ng/ml, 20 ng/ml, or 200 ng/ml TCDD in olive oil. The injection hole was sealed with melted paraffin. Following injection, the eggs were placed in egg flats, incubated at 100°F at 60% relative humidity, and rotated every two hours. The incubator, fabricated entirely of stainless steel at Clemson University for 2,3,7,8-TCDD work, was equipped with high and low temperature alarms and maintained the set temperature to within 0.5 °F.

In-ovo exposure study

Reproductively active adult chickens were dosed i.m. twice weekly for six weeks with either 0, 0.03, or 6 ug TCDD to simulate 10% of 0, 1ppb, or 200ppb TCDD in the feed, from control, post redmediation, and preremediation soils, respectively. At the end of the six weeks, immune and endocrine endpoints were assessed in the adults. After the first injection, the high dose (6 ug TCDD) began producing fewer eggs and after 10 days egg production stopped altogether. Due to this finding a cage of the high dose chickens were kept one month post-treatment (HPT) to determine if reproduction would recover[5]. Eggs laid during the six weeks were incubated, hatched, and grown to 14 days. At 14 days, the chicks were euthanized and immune and mixed function oxidase (MFO) endpoints were assessed. Sixteen chicks (8 control, 8 low dose) from the final hatch were allowed to grow to reproductive age to note onset of reproduction, egg production, and egg fertility[5]. At 30 weeks of age, these F_1 adults were euthanized and immune and MFO endpoints were assessed.

Analysis:

Lymphocyte Blastogenesis

T and B lymphocyte blastogenesis was determined by a modification of Lee[6]. Briefly, spleens were processed and diluted to $1.0x10^7$ cells/ml in RPMI 1640 (RPMI 1640 1x1L bottle, 10 ml 100x NEAA, 50 ml FBS, 10 ml sodium pyruvate, 1ml gentamyacin, 10 ml Hepes, 2.0g sodium bicarbonate, bring to vol. with ddH_20, pH 6.7-6.9). One hundred microliter aliquots of the resulting solution were dispensed into 96-well plates containing either 0, 2, 5, 20 ug/ml of Concavalin A (T cell) or 0, 100, 200, 400 ng/ml 12,13-Phorbol-Dibuterate (B cell). Each mitogen concentration was run in triplicate. Plates were incubated for 54 hours at 40-41°C and 5% CO_2. After 54 hours, 0.05 uCi of tritiated thymidine in 25 ul of RPMI 1640 was added to each well. The cells were harvested 12 hours later and counted in a Beckman liquid scintillation counter. Results were reported as percent of control (mitotic index).

Mixed Function Oxidase

Liver microsomes were prepared as described by Gard[7]. Ethoxyresorufin-O-dealkylase (EROD), pentoxyresorufin-O-dealkylase (PROD), methoxyresorufin-O-dealkylase (MROD), and benzoxyresorufin-O-dealkylase (BROD) assays were performed on the microsomes. Activity was determined by the methods of Prough et al.[8] and Burke et al.[9] respectively, which were modified for use in a 96-well fluorometric plate reader as reported previously[7]. Slight species specific modifications were made. Microsomes were diluted 20 fold for both EROD and MROD and five fold for BROD and PROD analysis. Samples were run in triplicate. The final concentrations ([FC]) of the substrates 7-ethoxy, 7-pentoxy, 7-methoxy, and 7 benzoxyresorufin used were $4.1x10^{-6}$, $3.6x10^{-5}$, $4.7x10^{-6}$, and $1.2x10^{-5}$, respectively. Fluorescence was read using a Perkin-Elmer LS 50 Luminescence Spectrometer that read each plate five times to obtain a kinetic determination of the reaction rate. The excitation wavelength was 530 nm and the emission wavelength was 585 nm. Protein concentration was determined using bicinchoninic acid[10]. Enzyme activities were expressed as picomoles of substrate dealkylated per mg protein per minute.

Statistics

The statistical design was determined to be a split-split plot due to the pens within treatments. In adults comparisons were made between the three dose levels {Dose}, and between the pens with in each dose {Pen(Dose)}. In the hatchlings comparisons were made between the dose levels {Dose}, between the dose level over the exposure time (Dose x Hatch) and between the pens with in each dose {Pen(Dose)}. Analysis was run using SAS with α=0.05. T and B cell data was log transformed to

normalize it. Type III sums of squares were analyzed. Significant differences were reported {Dose, Pen(Dose), and Dose x Hatch} and differences between hatches were determined by LS Means.

3. RESULTS

Egg Injection Study

At levels above 200 ppt no embryos survived to 14 days; therefore, immune endpoints were assessed only in the 0, 2, 20, and 200 ppt dose levels (Table 1). Both B and T cell proliferation exhibited stimulation over control at the two lowest doses and showed suppression at the highest dose. This, however, was not significant since the highest dose had an *n* value of one. Induction in both EROD and PROD was observed in these hatchlings and has been reported elsewhere[11].

In-ovo Exposure

Immune alterations were observed in both the adults and the hatchlings (Table 2). Since high dose reproduction stopped, immune function between only the low dose and control hatchlings was able to be measured. High post-treatment adult livers were destroyed in the freezer; therefore, MFO activity was not determined. Also, no proliferation data is available for hatch one chicks because the cell cultures grew out of their media before the addition of the radiolable. In addition, IgG values are not reported for hatchlings due to possible maternal transfer of this antibody.

Table 1. Summary of Immune Endpoint Results from the Egg Injection Hatchling Study.

Dose (ppt)	B cell Proliferation (MI)	T cell Proliferation (MI)
0 ppt (5)	.52 ± .52	.9 ± 1.0
2 ppt (6)	1.60 ± 1.18	1.7 ± 2.4
20 ppt (5)	.73 ± .63	4.6 ± 6.8
200 ppt (1)	.26	1.8

Adults exhibited T and B cell proliferation alterations that were significant by Dose. Hatchlings, however, exhibited significant differences between the low dose and control in B cell proliferation both by Dose and by Dose x Hatch. Low dose B cell proliferation exhibited parental exposure-responsive suppression over the exposure period, with the exception of hatch three which appears to be an anomaly. Although not significant, low dose hatchlings that were grown to 30 weeks were observed to still exhibit B cell proliferationlevels lower than control.

Adult hen mixed function oxidase (MFO) activity was observed to be significantly induced in a dose-dependent manner over control. Adult rooster EROD and PROD also exhibited a dose dependent increase that was significant, while MROD and BROD exhibited induction at the low dose and suppression at the high dose which were significantly different from control. Hatchling MFO activity at 14 days of age was also observed to increase in a dose dependent manner and in a exposure time dependent manner. PROD and BROD were noted to be less sensitive than EROD and MROD in the 14 day chicks; therefore, only EROD and MROD were assessed at 30 weeks. At thirty weeks of age the hatchlings were observed to exhibit both sex and dose dependent alterations in EROD and MROD activity

Table 2. Summary Of *In-Ovo* Exposure Immune Endpoint Results

Age	Hatch Week	Treatment (n)	B cell Proliferation (MI)	T cell Proliferation (MI)
Adult - hens	NA	Control (12)	61.1 ± 2.99	7.9 ± 13.3
	NA	Low (12)	5 4.1 ± 8.4	8.4 ±11.8
	NA	High (6)	2.53 ± 3.6*	33.9 ± 19.9 *
	NA	HPT (6)	3.18 ± .81*	130.4 ± 143 *
F_1 14 day	1	Control (5)	NM	NM
	1	Low (5)	NM	NM
	2	Control (5)	4.1 ± .94	5.8 ± 11.6
	2	Low (5)	4.6 ± 1.13*	27.9 ± 7.9*
	3	Control (5)	60.6 ± 15.6*	3.7 ± 6.5
	3	Low (5)	134.4 ± 43.3*	4.2 ± 9.7
	4	Control (5)	4.2 ± 1.3*	19.8 ± 6.5
	4	Low (5)	2.6 ± .84*	11.5 ± 2.3
	5	Control (5)	7.02 ± .3.13	1.5 ± 1.6
	5	Low (5)	2.6 ± 1.12	3.0 ± 9.1
	6	Control (5)	.67 ± .09*	NM
	6	Low (5)	.74 ± .15*	NM
F_1 30 weeks	NA	Control (6)	3.2 ± 1.7	25.5 ± 35.3
	NA	Low (6)	2.06 ± 1.2	44.27 ± 25.3

*Significant at p,0.05, NM=Not Measured, NA=Not Applicable

4. CONCLUSIONS

Although not significantly, 2,3,78-TCDD appears to stimulate the immune system at low doses as seen in the egg injection study. At slightly higher levels b cell functionality appears to be detrimentally effected in *in-ovo* exposure. This is contradictory to the expected t cell alterations from murine prenatal exposure. This functional humoral immune (HI) alteration occurs at parental dose levels as low as .3 ug TCDD (0.198 ug/kg 2,3,7,8-TCDD at the end of six weeks). This could have significant population effects in the wild since animals with a compromised immune system are more susceptible to bacterial and viral infections. Hatchlings, then, with a suppressed humoral immune system could be at a higher risk than those that have not been exposed. This has the potential to affect populations if the hatchlings are unable to survive to reproduce. As expected, MFO activity was found to be induced; however, what was not expected was the observed sex dependent difference in effects of *in-ovo* exposed chicks at maturity. This seems to indicate that *in-ovo* exposure to an average parental dose of 0.198 ug/kg 2,3,7,8-TCDD (0.3 ug twice a week for six weeks) results in MFO imprinting.

Table 3. Summary of *in-ovo* Mixed Function Oxidase Results.

Age	Hatch Week	Treatment (n)	EROD	MROD	PROD	BROD
Adult - hens	NA	Control (10)	15.2 ± 2.9	74.4 ± 47.9	14.7 ± 2.6	13.3 ± 12.3
	NA	Low (9)	291.1 ± 42*	201.9 ± 50.1*	235.6 ± 13.6*	66.9 ± 75.3
	NA	High (8)	825.5 ± 101*	504.5 ± 391*	744.7 ± 97.8*	184.7 ± 187*
	NA	HPT	NM	NM	NM	NM
Adult - roosters	NA	Control (2)	12.5 ± .23	109.1 ± 31.2	11.8 ± .45	32.9 ± 72.6
	NA	Low (2)	228.9 ± 39.6*	652.8 ± 84.4*	261.7 ± 15.1*	527.7 ± 592.1
	NA	High (1)	1004.6*	203.02*	765.1*	139.5*
	NA	HPT	NM	NM	NM	NM
F_1 14 day	1	Control (4)	89.43 ± 86.2	85.7 ± 61.2	1.81 ± .56	2.09 ± 1.5
	1	Low(4)	32.73 ± 18.4	78.0 ± 13.8	1.2 ± .68	2.78 ± 2.3
	1	High (4)	76.49 ± 34.6	67.5 ± 26.9	2.4 ± 2.8	21.36 ± 30.5*
	3	Control (4)	76.6 ± 29.6	773 ± 13.3	.98 ± .5	2.6 ± 1.0
	3	Low (4)	201.1 ± 101.1	114.9 ± 35.3	2.8 ± 1.7	15.6 ± 8.6
	6	Control (4)	122.7 ± 51.02	89.7 ± 66.6	.65 ± 1.3	.52 ± .74
	6	Low (4)	223.2 ± 79.95	131.6 ± 4.7	1.07 ± .06	0 ± .89
F_1 30 weeks-Hens	NA	Control (4)	.52 ± .1	12.6 ± 2.1	NM	NM
	NA	Low (4)	.4 ± .03	14.8 ± 3.4	NM	NM
F_1 30 weeks-Roosters	NA	Control (4)	.56 ± .16	31.5 ± 4.6	NM	NM
	NA	Low (4)	.82 ± 18	75.3 ± 12.6*	NM	NM

*Significant at p,0.05, NM=Not Measured, NA=Not Applicable

5. REFERENCES

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