Xanthohumol Effect on 2,3,7,8-Tetrachlorodibenzo-p-dioxin-Treated Japanese Quails in Terms of Serum Lipids, Liver Enzymes, Estradiol, and Thyroid Hormones

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ABSTRACT: Dioxins are compounds classified as persistent organic pollutants, from which 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is the most toxic to living organisms. TCDD is considered a carcinogen and has proinflammatory influence on animals and humans, promoting free radicals’ formation, and binding with the aryl hydrocarbon receptor (AhR) leads to expression of cytochrome p-450 genes that in turn predisposes to mutations. Natural flavonoids, in this case xanthohumol (XN), have been reported to attenuate TCDD toxicity through inhibition of the transformation of the AhR. Moreover, XN shows antioxidant properties. The aim of the study was to compare the effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin and XN on lipid, liver enzyme, estradiol, and thyroid hormone levels in the serum of Japanese quails. Adult, six-month-old, Japanese quails were divided into eight groups according to treatment procedures. Serum levels of total cholesterol (TCh), high-density lipoproteins (HDLs), triglycerides (TGs), estradiol, triiodothyronine, and thyroxine, and activities of alkaline phosphatase, aspartate aminotransferase, and alanine aminotransferase were measured. In comparison with the control group, 2,3,7,8-tetrachlorodibenzo-p-dioxin significantly decreased concentrations of serum HDLs and thyroid hormones and significantly increased the serum TCh level. Levels of serum TGs, liver enzymes, and estradiol were not changed after 2,3,7,8-tetrachlorodibenzo-p-dioxin treatment. Based on our data, XN treatment may also increase the levels of thyroid hormones. Moreover, the tested dioxin disrupts the liver function, especially changing lipids’ metabolism. Therefore, more studies are needed for better understanding the mechanism of toxic influence of 2,3,7,8-tetrachlorodibenzo-p-dioxin on key metabolic pathways and organs in living organisms.

1. INTRODUCTION

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is the most-toxic substance from the group of dioxins. Dioxins are compounds classified as persistent organic pollutants from which, most are considered as carcinogens and have proinflammatory influence on animals and humans as well. Dioxins are created in many technological processes that are based on the burning of organic matter at a temperature of 200−900 °C, in the presence of water and chlorine and at a low oxygen content. Despite legal restrictions established by the Stockholm Convention, a significant amount of dioxins is still produced and released into the environment, including in combustion diesel engines, engine oil leaks, and garbage dumps. The characteristic feature of dioxins, including TCDD, is stability under all environmental conditions, bioaccumulation and atmospheric or oceanic transport over long distances. The main sources of human and animal exposure to dioxins are food (over 90%) and polluted air. TCDD is toxic to living organisms and causes, for example, inflammation of tissues and organs.

The mechanism of toxic action is complex. First, TCDD promotes the formation of free radicals that directly destroy cell membranes by peroxidation of membrane lipids. Second, thanks to its ability to activate the cytoplasmatic aryl hydrocarbon receptor (AhR) and the aryl hydrocarbon receptor nuclear translocator, TCDD causes the migration of these receptors to the cell nucleus and, for example, induction of expression of cytochrome P-450 genes and other proteins involved in the metabolism of xenobiotics. Such action, in turn,
lipids showed significant effects on harmful changes caused by TCDD. More-\textemdash\textit{over, the TCDD-activated AhR interferes with the estrogen receptor function, thereby causing reproductive and behavioral disorders.\textemdash}\textit{Moreover, the most-known and well-described adverse effects of dioxins on human and animal organisms are chloracne, infertility, increased concentration of globulins, delayed immune response, and disruption in thyroid hormone concentration. Based on studies involving intoxication of various animals, TCDD may cause cancer, porphyria, hepatomegaly, atrophy of thymus, and bone hypoplasia and inhibit ovulation.}

\textit{Xanthohumol (XN), the most-abundant prenylated flavonoid extracted from hops, was shown to be an effective factor in the prevention of cardiovascular diseases and neurodegenerative diseases, and it has anticancer properties.\textit{It was demonstrated by in vitro and in vivo studies that XN inhibits metabolic syndrome processes, exerts anti-inflammatory, antimicrobial, and antioxidant activities, and could be a potential therapeutic agent for the prevention of colitis. This chemical may be also useful for the prevention of bone, liver, skin, and thyroid diseases.}}

\textit{Natural flavonoids, in this case XN, have been reported to attenuate TCDD toxicity through inhibition of the transformation of the AhR. XN also decreases the overexpression of cytochrome P450 1A1 (CYP1A1), which is a marker for AhR activity. Moreover, antioxidant properties of XN have been shown, which may be the alternative way of its protective role in dioxin intoxication.}}

\textit{In our research, we examined the influence of XN on TCDD-intoxicated organisms, taking into account changes in serum concentration of estradiol, thyroid hormones, and liver function indicators. We have chosen those parameters based on data indicating that antioxidant properties of XN may have beneficial effects on harmful changes caused by TCDD. Moreover, estrogens are synthesized from cholesterol, undergo a number of chemical reactions in liver, influence the serum level of high-density lipoprotein (HDL) and low-density lipoprotein (LDL), and increase the synthesis of endogenous proteins including thyroid hormone-binding proteins.}}

\textit{Thyroid hormones, in our case free triiodothyronine (FT3) and free thyroxine (FT4), are involved in fatty acid metabolism and also influence gonadal function. The liver is the main organ responsible for the metabolism of toxin, fatty acid, lipid, and protein transformations, and therefore, measuring the level of its main enzymes is crucial for determining the influence of toxins and their potential antagonists on many metabolism paths in living organisms.}}

\textit{There is a lack of information about the linking influence of both TCDD and XN, on birds, which can be further related to humans. In the present study, we examined the effects of TCDD and XN on circulating lipid, liver enzyme, estradiol, and thyroid hormone levels in the serum of Japanese quails.}}

\textbf{2. RESULTS}

\textbf{2.1. Effects of TCDD and XN on Serum Lipids and Liver Enzymes.} The birds did not show any overt signs of TCDD toxicity like, for example, weight loss. Analysis of serum lipids showed significant differences in levels of serum total cholesterol (TCh) ($p = 0.011$) and serum HDL ($p < 0.0001$) between tested groups (Figure 1 and Figure 2, respectively). Intergroup comparisons revealed a significant increase of TCh concentration in group 4 (234.1 ± 41.3 mg/dL) and group 7 (237.9 ± 56.8 mg/dL) in comparison to group 1 (177.8 ± 45.4 mg/dL) and group 3 (177.3 ± 36.9 mg/dL) ($p < 0.05$ for all) (Figure 1). We observed a significant decrease of serum HDL concentration in group 4 (16.7 ± 6.1 mg/dL), group 5 (12.2 ± 4.2 mg/dL), group 6 (12.1 ± 5.8 mg/dL), group 7 (13.7 ± 7.3 mg/dL), and group 8 (9.8 ± 4.1 mg/dL) as compared to group 1 (22.6 ± 9.9 mg/dL) ($p < 0.05$ for all). Significantly lower levels of serum HDL in group 8 than in group 2 (17.0 ± 5.7 mg/dL) and in groups 5–8 than in group 3 (21.7 ± 4.5 mg/dL) were shown ($p < 0.05$ for all, Figure 2).

\textit{No significant effects on the concentration of serum triglycerides (TGs) were demonstrated ($p = 0.108$, Figure 3). No statistically significant differences were observed in the activities of liver enzymes between the tested groups (for alkaline phosphatase (ALP): $p = 0.239$; for aspartate aminotransferase (AST): $p = 0.149$; for alanine aminotransferase (ALT): $p = 0.410$, Figures 4–6).}

\textbf{2.2. Effects of TCDD and XN on Estradiol and Thyroid Hormones.} We did not observe a significant difference in...
serum levels of estradiol between the tested groups ($p = 0.786$, Figure 7). Analysis of the results of thyroid hormones showed significant differences in the concentration of serum FT3 ($p = 0.0008$) and serum FT4 ($p < 0.0001$) between groups (Figures 8 and 9, respectively). Intergroup comparisons demonstrated a significantly lower level of FT3 in group 7 ($2.31 \pm 0.32$ pg./mL) as compared to control group 1 ($2.82 \pm 0.32$ pg./mL), group 2 ($2.74 \pm 0.66$ pg./mL), group 3 ($2.94 \pm 0.21$ pg./mL), and group 4 ($3.02 \pm 0.21$ pg./mL) ($p < 0.05$ for all, Figure 8). The concentration of serum FT4 significantly decreased in group 4 ($0.42 \pm 0.14$ ng/mL), group 5 ($0.45 \pm 0.15$ ng/mL), and group 7 ($0.48 \pm 0.19$ ng/mL) in comparison to control group 1 ($0.79 \pm 0.42$ ng/mL) ($p < 0.05$ for all). On the contrary, serum FT4 concentration was significantly higher in group 6 ($0.85 \pm 0.21$ ng/mL) and group 8 ($0.78 \pm 0.31$ ng/mL) in comparison to group 4 and group 5 ($p < 0.05$ for all, Figure 9).

3. DISCUSSION

To our knowledge, we are the first to examine the effect of XN on TCDD-induced toxicity in Japanese quails’ model. The vast majority of research in this area is conducted on rats or mice. We have decided to perform all of our experiments on Japanese quails (Coturnix japonica) because quails have near similar physiology to higher class animals (including humans) so the results can be applied for higher class animals or
Figure 8. Concentration of the triiodothyronine fraction (FT3) in the serum of the tested groups. The letters indicate statistically significant differences in intergroup comparisons (posthoc LSD test, p < 0.05): a: gr.7 vs gr.1,2,3,4; b: gr.4 vs gr.6,8; c: gr.5 vs gr.6,8.

Figure 9. Concentration of the thyroxine fraction (FT4) in the serum of the tested groups. The letters indicate statistically significant differences in intergroup comparisons (posthoc LSD test, p < 0.05): a: gr.1 vs gr.4,5,7; b: gr.4 vs gr.6,8; c: gr.5 vs gr.6,8.

Humans. They are also more disease-resistant than other forms of poultry, and their response to laboratory manipulation, including toxicology research, is very good because of the high metabolic rate.81-83

We observed a significant increase in the serum TCH level in quails treated with TCDD in concentrations of 0.5 μg/kg body mass and 2.0 μg/kg body mass, which correspond to the results obtained in previously performed experiments on a rat model.8 On the other hand, a previous study by Zhang et al.44 reported the lack of significant changes in the levels of lipids and lipoproteins between mice treated with dioxins and those not treated with dioxins. In contrast, studies by Angrish et al.8 and Fader et al.45 have reported a lack of change in the levels of serum TGs in mice. In contrast, serum TG concentration was elevated in fed mice treated with TCDD but remained unchanged in fasted mice, which suggested that the access to animal feed affects the lipidemic profile.45

TCDD treatment did not affect serum ALP, AST, and ALT activities. Similar to our results, also the study by Fouzy et al.4 showed a lack of significant changes in the activity of serum AST and ALT in female goats treated with TCDD as compared to the untreated group, but the activity of ALP was significantly increased. On the other hand, Ohbayashi et al.46 reported significantly increased release of serum AST in the group of female rats treated with a high dose of TCDD (30–300 μg/kg body mass).

On the basis of our results, we suppose that the level of lipids and liver enzyme function appear to be less sensitive to TCDD in Japanese quails than in a chicken or mammal model. XN treatment did not influence these parameters in quail sera. We suggest also that in quails, TCDD-mediated hepatic system damage is not as intense as in mammals. Additional studies are needed to verify this hypothesis.

Our study showed no significant differences in the serum estradiol level between the tested groups. Sechman et al.51 showed an inhibitory effect of TCDD on the estradiol level in the in vitro study of chicken ovarian follicles. The authors indicated that TCDD influences ovarian steroidogenesis and inhibits sex hormone secretion. We observed the decrease in the estradiol level in serum after injections of XN, but these results were not significant. Additional studies ought to be performed to evaluate the effect of XN on estradiol secretion in quails' model.

Numerous studies proved that TCDD affected the levels of serum FT3 and FT4 in rodents52-54 and humans.55 The results of our experiment showed that TCDD significantly decreased serum FT4 and FT3 concentrations, especially in groups treated with TCDD in a concentration of 2.0 μg/kg body mass. These data are in agreement with the study by Katarzynska et al.56 in which the decrease in FT4 and FT3 levels in the serum of the laying chicken was presented. Studies

livers.47 There are few studies on the TCDD toxicity to quails' organisms. Studies by Farmahin et al.48 showed that the binding affinity of TCDD to avian AhR1 was slightly higher than that of another dioxin in Japanese quails.

An analysis of serum lipid composition in quails showed that TCDD treatment decreased the level of the HDL fraction. Our results are consistent with those obtained by Tanos et al.46 and Angrish et al.,5 where a decrease in HDL and LDL fractions was observed in the serum of mice, rats, and guinea pigs. The authors suggested that these changes are driven by AhR-mediated abnormalities in liver lipid metabolism, which reduce the formation of apolipoproteins and disrupt the transport of lipid fractions.8 Alterations of these processes are reflected in the reduction in the secretion of serum lipoproteins.

We observed that the lowest concentrations of serum HDL were measured in TCDD+XN-treated quails (groups 5, 6, 7, and 8). This result suggests that the alleviation by XN of TCDD toxicity is response-dependent, and XN may actually exacerbate some effects of TCDD.

Our study showed that the levels of serum TGs were not altered among the tested groups. A previous study suggested that TCDD induces adipose lipolysis in rats.59 It may be possible that mobilization of peripheral fat stored in adipocytes increased the serum TG level. On the other hand, Angrish et al.5 and Fader et al.45 have reported a lack of change in the levels of serum TGs in mice. In contrast, serum TG concentration was elevated in fed mice treated with TCDD but remained unchanged in fasted mice, which suggested that the access to animal feed affects the lipidemic profile.45

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by Kato et al.\textsuperscript{52,53} also provided evidence that serum FT4 was markedly decreased in mice after treatment with polychlorinated biphenyls that belong, with TCDD also, to the so-called “persistent environmental pollutants”. It was reported that the decrease in the serum FT4 level is associated mainly with the increase in thyroxine accumulation in the liver and partially with the increased excretion of biliary thyroxine metabolites and/or with the development of liver hypothyroidism.\textsuperscript{52,53}

Another possible explanation of the changes in FT4 and FT3 concentrations in blood was proposed by Webb and McNab\textsuperscript{57} and confirmed in the study by Katarzynska et al.\textsuperscript{56} In birds, TCDD can directly affect thyroid hormone biosynthesis and secretion, and as a consequence, it can disrupt the endocrine function of the thyroid gland.

We demonstrated that in TCDD+XN-treated groups, serum levels of FT4 and FT3 are at a similar level to the control group but in groups treated with TCDD alone, levels of those parameters are lower than those in the control group. These results suggest that XN might act as a protective agent against TCDD toxicity. Our findings are consistent with the studies of Radovic et al.\textsuperscript{58} who demonstrated the significant role of XN in the thyroid hormone metabolism and distribution by modulation of expression of rat liver enzymes. Previous studies revealed that XN stimulates the uptake of iodide in rat thyrocyte cells, which can be made use for more-efficient radiiodine therapy of the thyroid.\textsuperscript{58}

In conclusion, examination of the impact of XN on TCDD-induced toxicity in Japanese quails’ model is innovative. TCDD causes an increase in the serum TCH level in the studied organisms. On the other hand, quails are less sensitive to TCDD than chickens or some mammals, despite the fact they are considered as a good model for toxicological studies.\textsuperscript{59} Further research is needed to examine the antioxidant mechanism of action in reducing negative effects of TCDD-induced inflammatory reactions in tissues and organs that interfere with the working of organs and cell metabolism.

4. MATERIALS AND METHODS

4.1. Animal Handling and Treatment. Adult (six months old) female Japanese quails (C. japonica) with a weight of 160 g ± 10 g were housed in a 25 °C environment with 30–40% humidity and a 12/12-h light/dark cycle (7 AM to 7 PM). On day one of the experiment, the quails were randomly divided into eight groups according to treatment procedures: group 1 (n = 7) – control with an injection of 1% dimethyl sulfoxide (DMSO); group 2 (n = 7) – with an injection of XN, 10 mg/kg body mass; group 3 (n = 7) – with an injection of XN, 20 mg/kg body mass; group 4 (n = 15) – with a single injection of TCDD, 0.5 μg/kg body mass; group 5 (n = 12) – with a single injection of TCDD, 0.5 μg/kg body mass, and with subsequent injection of XN, 10 mg/kg body mass; group 6 (n = 12) – with a single injection of TCDD, 0.5 μg/kg body mass, and with subsequent injection of XN, 20 mg/kg body mass; group 7 (n = 15) – with a single injection of TCDD, 2.0 μg/kg body mass; and group 8 (n = 14) – with a single injection of TCDD, 2.0 μg/kg body mass, and with subsequent injection of XN, 20 mg/kg body mass. TCDD was intramuscularly injected in a single procedure; XN was administered to quails by intramuscular injection every 7 days (three times). Doses of TCDD and XN were developed based on previous studies\textsuperscript{5,13,20} and our own unpublished experimental data.

4.2. Ethical Statements. All quail handling and treatment procedures were approved by the Local Bioethics Council for Animal Experiments in Wroclaw (permission no. 52/2015). The permission was based on the European Union guidelines (Directive 2010/63/EU for animal experiments), and the experiment conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publications No. 86–26, revised 2014). All efforts were made to minimize animal suffering.

4.3. Serum Collection and Laboratory Analyses. On day 21, both were euthanized by decapitation, and blood samples were collected. Blood was clotted 30 min at room temperature and centrifuged (800g, 10 min. Room temperature) in an MPW-260R laboratory centrifuge (MPW Med. Instruments, Warszawa, Poland). Serum samples were divided into Eppendorf tubes, flash-frozen, and stored at −20 °C. Concentrations of TCh, HDL, and TGs in serum were determined using commercially available kits (Aqua-med, Łódź, Poland) according to the manufacturer’s protocol. Serum activities of ALP, AST, and ALT were measured spectrophotometrically, using commercial diagnostic kits (Aqua-med, Poland) and a Konelab 60i biochemical analyzer (Thermo Fisher Scientific, USA) at the Diagnostic Laboratory. Concentrations of estradiol, thyroxine fraction (FT4), and triiodothyronine fraction (FT3) in serum were determined using an Immulite 2000 Immunoassay System (Siemens Healthcare, Erlangen, FRG).

4.4. Statistical Analysis. Data were analyzed using Statistica 13.3 software (StatSoft, Tibco Software Inc., CA, USA). The distribution of data was analyzed by the Shapiro–Wilk normality test, and equality of variances by Levene’s test. Descriptive data were presented as mean, standard deviation (± SD), and 95% confidence interval (± 95% CI). Analysis of variance and posthoc Fisher’s LSD test were used for analyses of independent samples. Differences between treatment groups were considered significant when p ≤0.05.
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Notes
The authors declare no competing financial interest.

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ABBREVIATIONS
AL, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; TCH, total cholesterol; TG, triglycerides; XN, xanthohumol

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