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The Effect of TCDD Dioxin on the Rat Liver in Biochemical and Histological Assessment

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Eighteen male Wistar rats were divided into 3 groups of 6 animals each. Two groups received different intraperitoneal doses of TCDD (0.75 and 8μg) in DMSO solution and the third group (control) received only DMSO on days 0, 7 and 14. On day 21 the animals were sacrificed, and then blood tests, pathological examination and CYP1A1 activity measurement were performed. In rats that received a high dose of dioxin (8 μg) hepatic lobules revealed parenchymal degeneration and vacuolization of hepatocytes was observed, and also an increased CYP reaction was found in central parts of lobules, around the central vein. The reaction in control and low dose groups was weak. The resorufin level was significantly ($P<0.05$) higher in the group receiving a low dose of dioxin as compared to the control group. The study confirmed that TCDD damages the rat liver in a dose-dependent manner. Administration of high TCDD doses causing major liver damage also damaged CYP1A1 (based on higher resorufin levels in epiluminescence). TCDD activates CYP1A1, which was confirmed by increased immunohistochemical reactivity of central areas of hepatic lobules.

Key words: Rat pathology, CYP1A1, immunohistochemistry, liver, TCDD.

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Dynamic economical and industrial development lead to environmental pollution and contamination with increasing amounts of noxious agents. Among toxins, dioxins are particularly dangerous for living organisms. They belong to aromatic compounds with two central oxygen atoms bridging two benzene rings. Dioxins are the most potent known toxins. The most harmful of them is 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) being approximately 10 000 more toxic than potassium cyanide (SWEENEY *et al.* 2000). Major sources of environmental dioxin pollution are herbicide and fungicide manufacturing, the paper and cellulose industry, thermal reactions of chlorinated aromatic compounds and transformer or condenser break-down (BAUER *et al.* 1961; SILBERGELD 1995). The noxious effect of TCDD on humans was first reported in 1949 after a trichlorophenol reactor explosion in Nitro, West Virginia, USA (BAUER *et al.* 1961). Humans are exposed to dioxins present in food products or in the environment. Fats, milk, milk products, and fishes are the main sources of food dioxins (BAUER *et al.* 1961).

In humans, exposure to TCDD may result in skin lesions similar to acne (chloracne) and may also cause hepatomegaly. A transient increase in alanine and aspartate aminotransferases (ALT and AST) as well as gamma-glutamyl transpeptidase (GGTP) activities was observed in peripheral blood serum (DICKSON *et al.* 1993; STASKAL *et al.* 2005). TCDD is lipid-soluble and thus accumulates in adipose tissue. Dioxins may cause delayed effects that may be revealed several years after exposure to the toxin. Data on the noxious effects of TCDD on the nervous system are equivocal. Some authors report peripheral polyneuropathy, person-

ality and mood disorders observed after exposure (PEPER *et al.* 1993; WEBB *et al.* 1986). Similarly, ambiguous data concern the effect of TCDD on the endocrine system, but it seems plausible that contact with TCDD in doses investigated in humans has no effect on this system (PELCLOVA *et al.* 2001; PEPER *et al.* 1993; SWEENEY *et al.* 2000). Dioxins are potent mutagens causing DNA damage. They also exhibit strong teratogenic and carcinogenic properties (MCKEOWN-EYSSEN *et al.* 2004).

Isoenzymes of cytochrome P450 (CYP) constitute a system of hemoprotein microsomal enzymes responsible for the metabolism of a number of exogenous and endogenous substances in humans and in other mammals (CHANG *et al.* 1999). Cytochromes are located mainly in membranes of smooth endoplasmic reticulum. Currently, over 270 CYP families are known, including 18 in mammals. Until now over 50 CYP genes and over 30 CYP pseudo-genes have been described, classified into 18 families and 42 subfamilies (CLARKE 1998; NEBERT *et al.* 2002). The cytochrome P450 system is mainly connected to hepatic metabolism. It has been estimated that CYP1, CYP2 and CYP3 families constitute approximately 70% of hepatic CYP isoforms. These three families are involved in the majority of drug metabolic pathways in humans. Although CYP isoenzymes are mainly located in the liver, they have also been found in some other organs including brain, lung, kidney, pancreas, endocrine glands, testis, small intestine, bone marrow and skin (CHANG *et al.* 1999).

Cytochromes constitute the main enzymatic mechanism responsible for interactions of various chemical compounds. The CYP system takes part in metabolism of both exogenous and endogenous substances. It also plays a role in the pathogenesis of several diseases in which enzyme activity varies from optimum, too high or too low. This may be a result of genetic polymorphism due to an inherited unfavorable set of alleles, mutation of the enzyme encoding gene, effects of chemical substances on the CYP system or, finally, coincidence of the above factors (CLARKE 1998; GAMBLE *et al.* 2002; IBA *et al.* 1999).

The aim of the study was to assess the toxic effect of TCDD on rat liver, particularly doses of TCDD that can activate and damage proteins of CYP1A1.

Material and Methods

Animals and administration of TCDD

*Eighteen male Wistar rats aged 9 months and selected for equal weight of 180 g were used in the study. They were kept under controlled light conditions (LD 12:12 h, L 08.00 to 20.00 h) and fed an ordinary laboratory diet. The animals were handled according to the approved national guidelines for animal care. 2,3,7,8-tetrachlorodibenzo-p-dioxin (Cerillant, Inc., Austin, Texas, USA) in DMSO solution was applied as an inducing agent. Rats were divided into 3 groups of 6 animals each. Two groups received a different intraperitoneal dose of TCDD (0.75 μg and 8 μg) in 0.5 ml DMSO solution, whereas the third (control) group received only DMSO on days 0, 7 and 14. The rats were killed on day 21.

Clinical observations and biochemical blood parameters

The animals were observed every day for clinical signs. On day 21 the rats were killed by a pentobarbital overdose, the blood samples were taken from abdominal aorta and the activities of ALT, AST, GGTP, alkaline phosphates (ALP) and total bilirubin levels were assessed in blood serum using an Automatic Analyzer (Hitachi 917 Modular P analyzer, Hitachi, Ltd., Tokyo, Japan).

Pathological examinations and CYP1A1 activity measurement

The liver was examined for macroscopic lesions, then the liver samples were taken and fixed for 17 hours in 4 % buffered paraformaldehyde, washed in PBS (0.01 M; pH=7.4) and transferred to a 25 % sucrose solution with 0.01 % NaN_3. Tissue blocks were snap-frozen and 10 μm-thick sections were cut in a cryostat. Cryosections were mounted on poly-L-Lysine coated slides and air-dried. For the indirect immunofluorescence procedure, sections were hydrated and subsequently preincubated for 40 minutes in a solution containing 5% non-immune goat serum. Samples were then incubated overnight at room temperature with primary rabbit antibodies against CYP1A1 (Chemicon, Temecula, CA, # AB1247; diluted 1:500). The slides were subsequently washed in PBS and incubated for 1.5 hours with Cy3-conjugated goat anti rabbit antibody (Jackson IR, West Grove, PA, #111-165-144; diluted 1:500). Both primary antibodies and secondary antisera were diluted in the solution previously used for preincubation. After a final rinse in PBS, the samples were mounted in glycerin/PBS (3:1) at pH 8.6 (WALKER *et al.* 1998).

Additional liver samples were also subjected to routine hematoxylin-eosin staining and analysed by an independent pathologist.

*The experiments were approved by the Ethics Committee of the Jagiellonian University.

Table 1

Doses of TCDD administrated in studied groups of rats

Groups	Day of TCDD administration			
	Day 0	Day 7	Day 14	Day 21
Control group receving only DMSO n=6	0.5 ml DMSO	0.5 ml DMSO	0.5 ml DMSO	killed
Group 1 receving 0.75 μg TCDD n=6	0.75 μg TCDD in 0.5 ml DMSO	0.75 μg TCDD in 0.5 ml DMSO	0.75 μg TCDD in 0.5 ml DMSO	killed
Group 2 receving 8 μg TCDD) n=6	8 μg TCDD in 0.5 ml DMSO	8 μg TCDD in 0.5 ml DMSO	8 μg TCDD in 0.5 ml DMSO	killed

DMSO – dimethyl sulfoxide
TCDD – 2,3,7,8-tetrachlorodibenzo-p-dioxin

CYP1A1 activity was measured at the level of calculated activity as 7-ethoxyresorufin-o-deethylase (EROD) activity and at the protein level using an enzyme linked immunosorbent assay (ELISA), as described by ORIZ-DELGADO *et al.* (2008).

Statistical analysis

A one-way analysis of variance (ANOVA) was performed to investigate between-group differences. Whenever the variance analysis revealed statistically significant differences between the groups, Duncan's multiple comparison test was applied *post-hoc* to further define the differences (see Table 1 for results).

Results

Clinical observations and biochemical blood parameters

We observed no abnormal clinical signs and no macroscopic lesions of the liver in any of the groups of the rats.

Blood AST, GGTP and total bilirubin levels were significantly higher ($P<0.05$) in rats which received 8 μg of TCDD compared to control group and to rats receiving 0.75 μg of TCDD. No significant differences of blood ALT and ALP levels were found (Table 1).

Histology

No histological changes were found in the livers of rats in the control group and in the group receiving a low dose of dioxin (0.75 μg).

In rats receiving a high dose of dioxin (8 μg) hepatic lobules revealed parenchymal degeneration and vacuolization of hepatocytes. Moreover, the number of mitoses was higher and lipid deposits were found in macrophages together with early signs of hepatocyte steatosis.

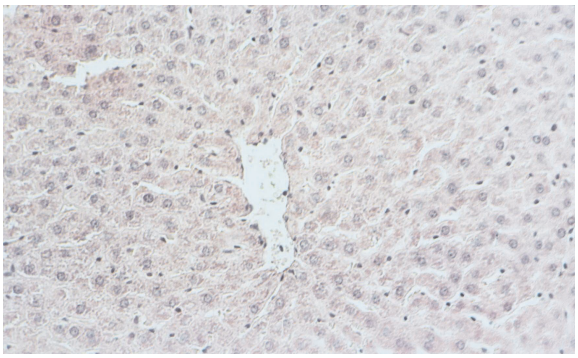

Fig. 1. Low dose (0.75 μg) dioxin group. Hepatic lobule showing regular-shaped hepatocytes, most of which have a single nucleus. Browicz-Kupffer cells are induced and easily visible within sinuses (HE ×200).

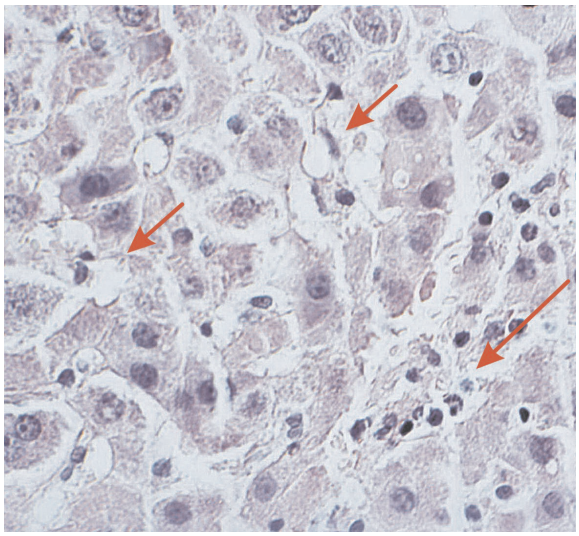

Fig. 2. High dose (8 μg) dioxin group. Small arrows – hepatocyte vacuolar changes surrounding lobular vein. Large arrow – inflammatory infiltrations surrounding hepatocytes undergoing necrotic changes (HE ×400).

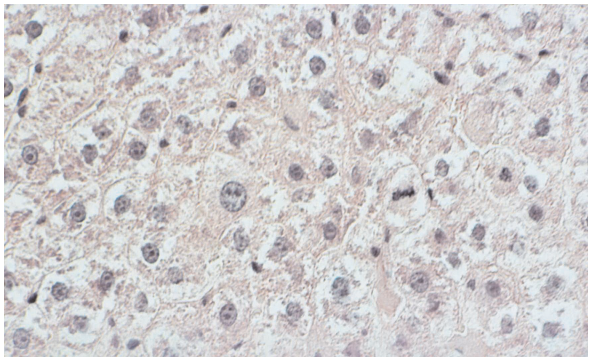

Fig. 3. High dose (8 μg) dioxin group. Parenchymal degeneration of hepatocytes. Empty spaces appear between the cells (dissociation). Hepatocytes are enlarged and their cytoplasm contains numerous granules caused by the disorganization of cytoplasmatic structures. Numerous mitoses are also visible (HE ×400).

Epiluminescence – CYP1A1 detection using immunohistochemical techniques

Weak expression was detected in both the control group and in the group of rats receiving a low dose of dioxin (0.75 μg). In rats receiving a high dose of dioxin (8 μg), an increased CYP reaction was found in the central parts of lobules, around the central vein.

Resorufin

The resorufin level was significantly ($P<0.05$) higher in the group that received a low dose of dioxin in comparison to the control group; no such difference was found between the high dose group and control. Moreover, the level of resorufin was significantly higher in the group receiving a low dose of dioxin than in the high-dose group. Mean resorufin levels in the groups are compared in Table 2.

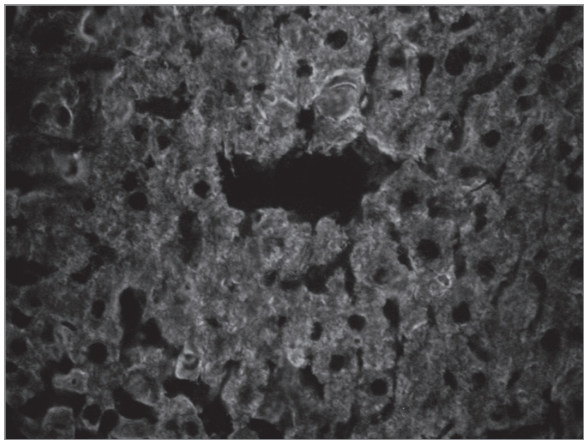

Fig. 4. CYP1A1 immunohistochemistry in the liver of control group animals. Moderate and rather weak expression of cytochrome in hepatocytes.

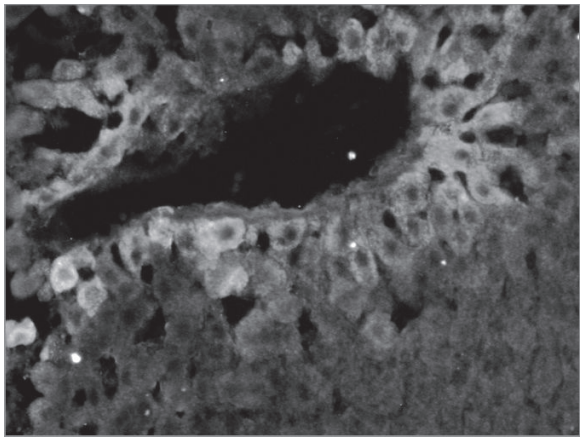

Fig. 5. CYP1A1 immunohistochemistry in the liver of rats receiving a high dose of dioxin (8 μg). Increased cytochrome immunostaining in hepatocytes around the central vein.

Table 2

Mean blood biochemical parameters. In each group n=6

Groups	ALT		AST		GGTP		ALP		Bilirubin	
	Mean (IU/l)	SD	Mean (IU/l)	SD	Mean (IU/l)	SD	Mean (IU/l)	SD	Mean (IU/l)	SD
Control DMSO	130.7	25.9	199.3	55	0	–	299.8	47	3.36	0.5
Group 1 0.75 μg TCDD	108.4	48.5	214.9	84.3	0.33	0.5	351.7	41.3	3.6	0.7
Group 2 8 μg TCDD	94.3	20.3	441.6	236.7	5.3	3.7	479	217.2	13.7	10.6

ALT – alanine aminotransferases
ALP – alkaline phosphates
AST – aspartate aminotransferases
DMSO – dimethyl sulfoxide
ELISA – enzyme linked immunosorbent assay
GGTP – gamma-glutamyl transpeptidase
TCDD – 2,3,7,8-tetrachlorodibenzo-p-dioxin

Table 3

Mean resorufin levels in respective groups of rats. In each group n=6

Rats	The mean value of resorufin (EROD) forming at 100 μg protein during 1 minute	Standard deviation
Control DMSO	0.13	0.07
Group 1 0.75 μg TCDD	1.33	0.38
Group 1 8 μg TCDD	0.21	0.11

DMSO – dimethyl sulfoxide
EROD – 7-ethoxyresorufin-o-deethylase
TCDD – 2,3,7,8-tetrachlorodibenzo-p-dioxin.

Discussion

Most animal studies on TCDD effects concern chronic administration of this substance. For this reason in our study we have chosen to administer TCDD in higher doses and a shorter time. Moreover, the majority of studies included female rats, as female sex was related to tumors resulting from exposure to TCDD. Our study, however, was not intended to investigate tumors, but to assess effects of TCDD on biochemical parameters and liver histology, as well as CYP activity.

The changes in liver function tests observed in our study were caused by hepatic damage, consistent with other studies of TCDD effects in rats (LEE *et al.* 2005; OHBAYASHI *et al.* 2007; YAMASHITA *et al.* 1992). This is particularly clear in the comparison of the group receiving a low dose of TCDD and the control group, where the difference is small, as opposed to the comparison of the high-dose TCDD group and control group, where the difference is large. The damage is proportional to TCDD dose.

Histological assessment confirmed hepatic damage resulting from dioxin exposure. This has been proven in numerous animal models. The lesions are usually characterized by steatosis, necrosis and lobular fibrosis. An increased number of mitoses resulting from TCDD exposure may lead to development of tumors and precancer lesions. This effect is characteristic of carcinogenic compounds which, however, do not have genotoxic properties (MANN 1997). In addition to parenchymal and vacuolar degeneration, ROTKIEWICZ (2004) described mitochondrial damage resulting in enlargement of mitochondria, a lower number of cristae and expansion of Golgi apparatus (ROTKIEWICZ 2004). Changes on the endoplasmic reticulum (ER) were also visible. Rough ER undergoes degranulation and smooth ER channels become enlarged. The increased number of mitoses and degenerative lesions described by BUNTON *et al.* (1997) were also observed in our study (BUNTON *et al.* 1997; GOLDSWORTHY *et al.* 1991).

TCDD activates mainly CYP1A1, CYP1A2 and CYP1B located in the central part of the hepatic lobule. For immunohistochemical assessment of CYP1A1, WALKER *et al.* (1998) administered TCDD for a few days to one group of rats, and smaller doses of the compound for a longer time to another. In both groups they observed an increased immunohistochemical reaction in central parts of hepatic lobules. The study also revealed differences in CYP1A and CYP1B activation, which depends on the TCDD dosage. CYP1A is activated by smaller doses. The difference of dose response between CYP1A and CYP1B is a result of different intensity of cell response in CYP1A and CYP1B locations. The mechanism of CYP1A induction by TCDD is unknown, but it probably reflects differences in hepatocyte physiology in various regions of the liver.

A positive reaction was not recorded for neither the control group nor the low dose dioxin (0.75 μg) group. In the high dose group, an increased CYP reaction was found in the central hepatic lobules around the central vein. Moreover, the initial stages of hepatocyte steatosis were detected. The destructive effect of TCDD on the liver was confirmed by biochemical tests and histological assessment. We have administered very high TCDD doses to rats in order to examine if they cause additional CYP damage, which would result in lower immunohistochemical activity of the cytochrome. However, we did not detect decreased immunohistochemical activity of CYP1A1 in the range of very high doses used in our study.

Nevertheless, we have noted that low TCDD doses caused a statistically significant increase of CYP1A1 activity, as reflected by the resorufin level. This is quite a typical reaction of increased enzyme activity as a result of stimulatory (in this case) activity of a toxic substance, which activates hepatocytes to counteract the noxious effect of the toxin. On the other hand, administration of a high dose of TCDD caused a fall in CYP1A1 activity, clearly reflecting enzyme deactivation. This could be explained by an early stage of severe functional hepatocyte damage, although such extreme histological damage was not detected. It may be a result of CYP1A1 protein destruction caused by the toxic effect of TCDD.

In conclusion, the study confirmed that TCDD damages the rat liver in a dose-dependent manner. This was shown by both biochemical tests and histological assessment of the liver. The administra-

tion of high TCDD doses causing major liver damage also damaged CYP1A1 as reflected by increased resorufin levels in epiluminescence in rats exposed to a high dose of TCDD. It was shown that TCDD activates CYP1A1, resulting in increased immunohistochemical reactivity of the cytochrom in the central areas of hepatic lobules.

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