The effects of hexachloronaphthalene on selected parameters of heme biosynthesis and systemic toxicity in female wistar rats after 90-day oral exposure

Michal Klimczak | Adam Darago | Elzbieta Bruchajzer | Katarzyna Domeradzka-Gajda | Maciej Stepnik | Katarzyna Kuzajska | Anna Kilanowicz

1Department of Toxicology, Faculty of Pharmacy, Medical University of Lodz, Muszynskiego 1, Lodz 90-151, Poland
2Department of Toxicology and Carcinogenesis, Nofer Institute of Occupational Medicine, Sw. Teresy 8, Lodz 91-348, Poland

Correspondence
Adam Darago, Department of Toxicology, Faculty of Pharmacy, Medical University of Lodz, Muszynskiego 1, Lodz 90-151, Poland.
Email: adam.darago@umed.lodz.pl

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Abstract
Hexachloronaphthalenes (HxCNs) are the most toxic congeners of polychlorinated naphthalenes, a group of compounds lately included into the list of persistent organic pollutants (POPs). This study presents the effects of 90-day intragastric administration of HxCN to female Wistar rats at doses of 0.03, 0.1, and 0.3 mg/kg body weight. The study examined selected parameters of the heme synthesis pathway, oxidative stress, hepatic cytochromes level, and basic hematological indicators. A micronucleus test was also performed. The subchronic exposure of rats to HxCN resulted in disruption of heme biosynthesis, hematological disturbances, and hepatotoxicity. The highest dose of HxCN inhibited aminolevulinic acid dehydratase (ALA-D) and uroporphyrinogen decarboxylase (URO-D). Accumulation of higher carboxylated porphyrins in the liver and increased excretion of 5-aminolevulinic acid in the urine was observed after a dose of 0.1 mg/kg body weight. The most sensitive effect of HxCN in rats was very strong induction of hepatic CYP1A1 activity, which was observed after the lowest dose. The highest dose of HxCN induced significant thrombocytopenia, thymic atrophy and hepatotoxicity, expressed as hepatomegaly and hepatic steatosis.

KEYWORDS
CYP1A1, heme biosynthesis, hexachloronaphthalene, rat, toxicity

1 | INTRODUCTION

Polychlorinated naphthalenes (PCNs) are a group of aromatic compounds comprising 75 different congeners which persist throughout the environment, despite their production and use as dielectrics, lubricants, or plasticisers being ended. Even recently, they have been detected in urban and rural air, as well as in soil and sediments. PCNs were used as technical mixtures of different congeners (e.g. Hallowax) rather than single congeners. In addition, chlorinated naphthalenes were confirmed as impurities in commercial mixtures of PCBs (polychlorinated biphenyls), and they can be unintentionally formed in a number of industrial thermal processes. Occupational exposure by inhalation has also been reported in secondary nonferrous metallurgical facilities in China.
Persistent organic pollutants (POPs) are widely distributed throughout the environment and remain in it for very long time. Moreover, they accumulate in the fatty tissues of living organisms and are highly toxic not only for humans, but for the wildlife. PCNs also accumulate in human tissues (mainly body fat), as discussed by Fernandes et al.\(^9\) PCNs were lately (in 2015) included into the Stockholm Convention on POPs, which aims to protect human health and environment.\(^10\) It should be underlined that PCNs are still the least known group of compounds included into Stockholm Convention, whose data concerning toxicity are rather scarce. With the exception of some research papers regarding technical mixture of PCNs from the first half of the previous century, there are no data about subchronic or chronic exposure to chlorinated naphthalenes.\(^11\)

Chlorinated naphthalenes are more or less lipophilic planar compounds structurally similar to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), whose mode of action is mediated by the aryl hydrocarbon receptor (AhR), which may suggest dioxin-like toxicity.\(^11\) Hexachloronaphthalenes (HxCNs) appear to be some of the most toxic congeners, the most potent being PCN66 (1,2,3,4,6,7-hexachloronaphthalene), PCN67 (1,2,3,5,6,7-hexachloronaphthalene) and PCN70 (1,2,3,6,7,8-hexachloronaphthalene) (Figure 1), for which relative effect potencies seem to be approximately an order of magnitude higher than for some dioxin-like PCBs.\(^11\) PCNs, including HxCNs, cause signs characteristic of dioxin poisoning in experimental animals, such as body weight loss and hepatotoxicity,\(^12,13\) thymus atrophy,\(^12\) and reproductive and developmental toxicity.\(^14,15\) Moreover, these compounds induce enzymes of the cytochrome P450 family, especially CYP1A,\(^12,13\) which is also typical of dioxins.\(^16\)

Chlorinated aromatics, including dioxins, are known to significantly impair a range of processes, including heme biosynthesis in experimental animals.\(^17\) Such disruption may induce the accumulation and/or elimination of heme precursors, including 5-aminolevulinic acid (ALA), porphobilinogen (PBG) and porphyrins, resulting in porphyria. For example, by suppressing uroporphyrinogen decarboxylase (URO-D), TCDD causes an accumulation of uroporphyrins (UROs) in the liver and greater excretion of UROs in the urine.\(^17,18\) A literature review at the time of writing revealed no data about the influence of PCNs on heme biosynthesis.

Therefore, the aim of the present study was to make the first assessment of the effects of a subchronic (90-day) administration of HxCN to female rats on chosen parameters of the heme synthesis pathway, as well as on other systemic toxic effects, concerning oxidative stress, levels of hepatic cytochromes and basic hematology indicators. In addition, the study is the first to make a preliminary evaluation of the genotoxicity of HxCN using a micronucleus test.

2 | MATERIALS AND METHODS

2.1 | Chemicals

HxCN was synthesized according to Auger et al.\(^19\) and obtained from the Institute of Radiation, Faculty of Chemistry, Technical University of Lodz (Poland) as a mixture of HxCN congeners. This mixture was used in previous studies conducted in our department, its purity was 94.14% and it contained mainly PCN67 (81.17%), 5.85% accounted for heptachloronaphthalene, as was described earlier.\(^13,15,20\) The content of dioxins and furans, analyzed by isotope dilution HRGC/HRMS, was below 1 pg/mg.

2.2 | Animals and treatment

The experiment was performed on female Wistar rats from the breeding colony of the Medical University of Lodz. The animals had free access to water and food (standard pelleted diet Murigram—Agropol, Motycz, Poland) and were held at temperature of 22 ± 1°C and
humidity of 50 ± 5% with a light/dark cycle of 12 h/12 h. Body mass of animals, consumption of food and water were checked every day before administration. Moreover, general clinical observations of study animals were made daily. The investigation was approved by the Local Ethical Committee for Experimentation on Animals (Resolution No. 13/LB703/2014).

Each experimental group and control group consisted of 10 animals. HxCN was dissolved in sunflower oil and then administered intra-gastrically in repeated daily doses for a period of 90 days. The control rats obtained only sunflower oil in the same manner. The highest dose in this study—0.3 mg/kg body weight—was adopted from our previous developmental toxicity studies, where it appeared to be a LOAEL (lowest observed adverse effect level) for pregnant female rats. Other two doses accounted for one-third and one-tenth of the highest dose—0.1 mg/kg and 0.03 mg/kg body weight, respectively. Before the termination of the study, a 24 h urine collection was obtained from rats in order to analyze the concentrations of ALA and porphyrins. After the administration period, the animals were sacrificed by intracardiac puncture under light carbon dioxide narcosis. Blood, liver, kidneys, lungs, adrenals, heart, thymus, spleen, ovaries, uterus and both femora were collected for further assays.

2.3 | Assays

Hepatic and renal reduced glutathione (GSH) levels were determined spectrophotometrically via the reaction with 5,5′-dithiobis-(2-nitrobenzoic acid) as was described by Sedlak and Lindsay. Malondialdehyde (MDA) levels were determined spectrophotometrically after the reaction with thiobarbituric acid according to Uchiyama and Mihara. Serum total antioxidant status (TAS) was measured with use of a NX 2332 kit (Randox Laboratories, United Kingdom).

The total cytochrome P450 (CYP450) content of the hepatic microsomes was determined according to Omura and Sato as described by Guengerich and it was calculated from the sodium-dithionite-reduced carbon monoxide difference spectrum between 450 and 490 nm. The activity of CYP1A1 and CYP2B isozymes of cytochrome P450 was determined by measuring the fluorescence of resorufin formed by the O-dealkylation of 7-ethoxyresorufin and 7-pentoxyresorufin, which are substrates for CYP1A1 (EROD) and CYP2B (PROD), respectively, according to Burke et al. The level of cytochrome P450 and the activity of CYP1A1 and CYP2B were adjusted to microsomal protein concentration, which was determined according to Lowry et al.

The activity of aminolevulinic acid synthase (ALA-S, EC 2.3.1.37) was determined based on the spectrophotometric determination of pyrroles generated in the reaction of ALA with acetylacetone as was described by Sassa et al. The activity of aminolevulinic acid dehydratase (ALA-D, EC 4.2.1.24) was estimated according to Schlick et al. and based on spectrophotometric measurements of PBG generated under the influence of this enzyme. URO-D (EC 4.1.1.37) activity was assessed according to van Gelder et al. Briefly, after restoring the activity of the uroporphyrinogen synthase by the addition of dithiothreitol at 4°C, uroporphyrinogen formed from added PBG undergoes further oxidation under the influence of URO-D. Porphyrins formed in this reaction was determined as described below and URO-D activity was expressed as concentration of formed porphyrin [sum from heptacarboxyphorphyrin (HEPTA) to coproporphyrin (COPRO)] in nmol/mg of protein within 1 h.

The isolation of porphyrins from hepatic tissues was performed according to Luo and Lim. The obtained supernatant, as well as the urine samples, were examined using a chromatograph with a fluorescence detector (ACQUITY UPLC, Waters). Porphyrin separation was performed according to Benton et al. with modifications; a BEH C18 column (1.7 μm particle size, 2.1 × 100 mm, Waters) was used with a flow-rate of 0.35 mL/min at 35°C, an excitation wavelength (λex) of 404 nm and emission wavelength (λem) of 618 nm, and the following standards: Porphyrin Acid Chromatographic Marker Kit (Frontier Scientific) containing uroporphyrin I (URO), heptacarboxylic acid porphyrin I (HEPTA), hexacarboxylic acid porphyrin I (HEXA), pentacarboxylic acid porphyrin I (PENTA), coproporphyrin I (COPRO), and mesoporphyrin; isomers III of URO and COPRO were purchased from Frontier Scientific.

ALA concentration in urine was determined according to Oishi et al. and the separation of the ALA derivative formed in reaction with acetylacetone and formaldehyde was performed using a chromatograph with a fluorescence detector (ACQUITY UPLC, Waters) on a BEH C18 column (1.7 μm particle size, 2.1 × 100 mm, Waters) in isocratic flow (0.2 mL/min.) at 40°C. Methanol/water/acetic acid (50:50:1, v/v) was used as a solvent, λex = 370 nm and λem = 460 nm on fluorescence detector were applied.

2.4 | Micronucleus test

Bone marrow washed from the femoral bones was suspended in 2 mL prewarmed (37°C) bovine serum mixed with Sörensen’s buffer (1:1). The suspension was centrifuged (5 min, 1000 rpm) and smears were made after removing the supernatant. The slides were fixed with pure methanol (15 min) and stored at room temperature until analysis. Afterwards the slides were sequentially stained with non-diluted May-Grünwald stain for 3 min; May-Grünwald stain diluted with Sörensen’s Buffer (1:1) for 2 min; and Giemsa stain diluted with Sörensen’s Buffer (1:6) for 10 min. After washing and leaving for a few minutes on absorbent paper, the slides were soaked in 95% and 99% ethanol, for 1 and 8 min, respectively. The slides were analyzed under an Olympus Vanox AHBT3 light microscope using ×1000 magnification. The following cell types were counted: polychromatic erythrocytes (PCEs), mature normochromatic erythrocytes (NCEs), and micronucleated polychromatic erythrocytes (MPCEs). The proportion of PCes/NCEs was determined for each animal by counting at least 2000 erythrocytes for each bone marrow. For each animal, 4000 PCes were scored for the presence of micronuclei.

2.5 | Hematology

All hematological analyses were performed on the hematology analyzer and the following parameters were measured: white blood cell count...
(WBC), red blood cell count (RBC), RBC distribution width (RDW), hemoglobin (HGB), mean corpuscular volume (MCV), hematocrit (HTC), mean corpuscular hemoglobin (MCH), platelet count (PLT), and mean platelet volume (MPV).

2.6 | Histopathology

Liver, kidneys, lungs, adrenals, heart, thymus, spleen, ovaries, and uterus were weighed prior to fixation in 10% solution of buffered formalin. Histopathology examination was performed in the Department of Pathomorphology, Medical University, Lodz. Paraffin sections obtained by the routine method were stained with hematoxylin and eosin. Fatty changes in hepatocytes was confirmed by Oil Red staining on frozen sections of rat liver. An Olympus BX 41 microscope was used for histopathological observations.

2.7 | Statistical analysis

Statistical analysis was performed using SPSS ver. 25 (IBM). Levene’s test for the homogeneity of group variances was used, as well as the one-way analysis of variance (ANOVA). If the compared means were not identical, Tukey’s test was used as a post hoc test. In case of unequal variances a Welch test followed by Dunnett’s T3 test was applied. Difference between means was judged as statistically significant if \( P < .05 \).

 TABLE 1  Changes in body weight and organ weights following subchronic HxCN administration in female rats

| Parameters             | Dose (mg/kg body weight) |
|------------------------|--------------------------|
|                        | Control | 0.03 | 0.1  | 0.3   |
| Food consumed/rat/day (g) | 15.4 ± 1.25 | 13.6 ± 0.76 | 14.4 ± 0.94 | 14.5 ± 0.73 |
| Water consumed/rat/day (mL) | 32.5 ± 2.80 | 31.5 ± 2.03 | 34.7 ± 2.41 | 31.4 ± 3.15 |
| Body weight            |          |      |      |        |
| Initial                | 216 ± 5  | 210 ± 5 | 214 ± 7 | 226 ± 6 |
| Final                  | 248 ± 9  | 246 ± 9 | 238 ± 11 | 241 ± 3  |
| Liver weight           |          |      |      |        |
| Absolute               | 7.98 ± 0.34 | 7.73 ± 0.49 | 8.22 ± 0.61 | 12.27 ± 1.43\(^{abc}\) |
| Relative               | 3.22 ± 0.17 | 3.14 ± 0.15 | 3.45 ± 0.18 | 5.09 ± 0.55\(^{abc}\) |
| Kidneys weight         |          |      |      |        |
| Absolute               | 1.46 ± 0.06 | 1.47 ± 0.08 | 1.50 ± 0.07 | 1.55 ± 0.12 |
| Relative               | 0.62 ± 0.02 | 0.61 ± 0.03 | 0.62 ± 0.03 | 0.63 ± 0.05 |
| Lungs weight           |          |      |      |        |
| Absolute               | 1.26 ± 0.08 | 1.25 ± 0.11 | 1.28 ± 0.08 | 1.33 ± 0.14 |
| Relative               | 0.50 ± 0.04 | 0.51 ± 0.03 | 0.54 ± 0.02 | 0.55 ± 0.06 |
| Heart weight           |          |      |      |        |
| Absolute               | 0.72 ± 0.035 | 0.70 ± 0.056 | 0.69 ± 0.036 | 0.67 ± 0.029 |
| Relative               | 0.29 ± 0.008 | 0.29 ± 0.024 | 0.29 ± 0.006 | 0.29 ± 0.044 |
| Spleen weight          |          |      |      |        |
| Absolute               | 0.52 ± 0.083 | 0.50 ± 0.087 | 0.48 ± 0.039 | 0.55 ± 0.086 |
| Relative               | 0.21 ± 0.031 | 0.20 ± 0.032 | 0.20 ± 0.014 | 0.23 ± 0.038 |
| Thymus weight          |          |      |      |        |
| Absolute               | 0.25 ± 0.021 | 0.24 ± 0.037 | 0.20 ± 0.021 | 0.11 ± 0.013\(^{abc}\) |
| Relative               | 0.10 ± 0.011 | 0.10 ± 0.014 | 0.08 ± 0.012 | 0.05 ± 0.005\(^{abc}\) |
| Adrenals weight        |          |      |      |        |
| Absolute               | 0.056 ± 0.012 | 0.059 ± 0.013 | 0.047 ± 0.006 | 0.074 ± 0.005\(^{abc}\) |
| Relative               | 0.023 ± 0.005 | 0.024 ± 0.006 | 0.020 ± 0.002 | 0.031 ± 0.002\(^{abc}\) |
| Ovaries weight         |          |      |      |        |
| Absolute               | 0.094 ± 0.013 | 0.107 ± 0.028 | 0.096 ± 0.016 | 0.083 ± 0.007 |
| Relative               | 0.038 ± 0.004 | 0.043 ± 0.011 | 0.040 ± 0.008 | 0.034 ± 0.003 |
| Uterus weight          |          |      |      |        |
| Absolute               | 0.50 ± 0.034 | 0.56 ± 0.112 | 0.57 ± 0.133 | 0.38 ± 0.091 |
| Relative               | 0.20 ± 0.015 | 0.23 ± 0.043 | 0.24 ± 0.051 | 0.16 ± 0.039 |

All values expressed as means ± SD.

\(^{a}\)Significantly different from the control group, \( P < .05 \).

\(^{b}\)Significantly different from the 0.03 group, \( P < .05 \).

\(^{c}\)Significantly different from the 0.1 group, \( P < .05 \).
RESULTS

All female rats survived the administration period and no changes in the behavior or appearance were observed. No changes in consumption of food and water were noted throughout the whole experiment. All changes in body weight, as well as other organ weights, are presented in Table 1. There were no changes in absolute and relative weights of the kidneys, lungs, heart, spleen, ovaries and uterus, comparing to the control group. After subchronic exposure to HxCN at the highest dose (0.3 mg/kg), a significant increase in both absolute and relative liver and adrenals weights were observed. Conversely, weight of the thymus was significantly decreased.

Figure 2 presents the results of parameters relating to oxidative stress. After 90 days of HxCN administration, major changes were noted in hepatic MDA levels. The observed increase in MDA concentrations in the liver was dose dependent, amounting in rats treated with 0.3 mg HxCN/kg to over twice the control value. In addition, the highest HxCN dose reduced the level of TAS by about 21%. Levels of GSH seem to be unaffected by the administration of HxCN, as no differences were observed in any of the experimental groups.

Results referring to the levels of total cytochrome P450 (CYP450), CYP1A1, and CYP2B activities are presented in Figure 3. Subchronic exposure to HxCN triggered a very strong, dose-dependent, induction of CYP1A1 activity in liver and kidneys. The hepatic and renal activities of CYP1A1 in rats treated with 0.3 mg/kg HxCN in these organs were over 110-fold and 60-fold higher, respectively, compared to the control group. A similar, but not so spectacular effect was observed in CYP2B activity, whose activity rose 3-fold not only in liver, but also in the kidneys of rats following the highest HxCN dose. Interestingly, while the hepatic content of cytochrome P450 was significantly elevated in rats exposed to lower doses, those exposed to the highest dose demonstrated notably lower cytochrome P450 concentrations than both the control group and those administered with other doses of HxCN.

According to the potential influence of HxCN on heme biosynthesis, subchronic administration of the examined mixture at the highest dose was associated with a statistically significant decline in the activity of hepatic ALA-D and URO-D: around 40% and 30%, respectively (Figure 4). Although the activity of ALA-S in the liver and URO-D in the kidneys was also found to be diminished, these changes were statistically insignificant.

Exposure to HxCN had also an impact on concentration of porphyrins in the liver and urine (Figure 5). Hepatic uroporphyria was the foremost effect noted in rats administered with HxCN along with an elevated level of HEPTAs. Both were predominant origins of elevated total porphyrin concentration in the liver at the two higher doses of HxCN. Concentrations of hepatic porphyrins were over 2-fold and 5-fold greater than seen in the control group. No changes were noted concerning the remaining porphyrins, although the concentration of pentacarboxyporphyrins (PENTAs) was significantly diminished in the liver of rats administered with 0.1 and 0.3 mg/kg of HxCN.

In contrast to hepatic porphyrin concentration, administration of HxCN did not affect the levels of the higher carboxylated porphyrins (UROs and HEPTAs) in urine. Moreover, a remarkable decrease in total porphyrin concentration was found in the urine of rats treated with 0.3 mg HxCN/kg, and a significant decline in COPRO level was noted, with the concentration falling by over 6-fold (Figure 5). The concentration of PENTAs also decreased by around 3-fold. These effects were accompanied with an increase in ALA concentrations of around 3-fold with regard to the control group (Figure 6).
Histopathological examination revealed fatty degeneration in the livers of all female rats exposed only to the highest dose of HxCN (0.3 mg/kg), which was confirmed by oil red staining (Figure 7). In 6 out of 10 rat livers, mixed macrovesicular and microvesicular steatosis covering all lobule zones was observed in over 66% of hepatocytes. The remaining four livers were also characterized by a mixed steatosis, but in 33%–66% of hepatocytes in the central zones of the lobes. There were no changes in the kidneys, lungs, adrenals, heart, spleen, thymus, ovaries, and uterus.

A 90-day exposure to the highest dose of HxCN also had an impact on some hematological parameters. As is shown in Figure 8, lowered HGB, HTC, MCV and MCH values were observed mostly at the highest dose alone. Thrombocytopenia was another effect noted after administration of both highest doses: 0.1 and 0.3 mg/kg HxCN.

The results of the micronucleus test suggest that no dose of HxCN affected the formation of MPCEs, as roughly the same frequency of formation was found in all groups including the controls. In addition, the PCEs/NCEs ratio was invariant among the all groups (Table 2).

4 | DISCUSSION

The findings indicate that subchronic toxicity of HxCNs resulted in various effects, some of which resemble the effects of dioxins. One symptom typical of the toxic effects of dioxins and dioxin-like compounds is “wasting syndrome.” Although this effect was also evidenced for PCNs, it was not observed in the present study. This could be attributed to the low level of exposure.

Polyhalogenated organic compounds are considered to have hepatotoxic activity. Hepatomegaly, necrosis, and fatty changes occurred in experimental animals after exposure to TCDD. In short-term studies with HxCN, an increased relative liver mass was observed. Beyond this effect, Hooth et al. also noted hepatocellular diffuse fatty change after 2-week exposure to PCN66 (doses of 0.05 and 0.5 mg/kg) and focal to multifocal hepatocellular necrosis associated with acute inflammation after exposure to PCN67 (0.5 mg/kg). A subchronic administration of a 0.3 mg/kg dose of HxCN mixture in the present study containing both PCN66 and PCN67 caused marked hepatomegaly and steatosis, which resemble the effects of dioxin poisoning. Although these changes indicate a hepatotoxic action of HxCN, they are not indicative of porphyria in the liver. Another finding in this study concerning the highest dose, that is significantly decreased weight of the thymus, is also typical of animal exposure to TCDD and PCNs, including HxCNs.

Although several mechanisms have been proposed for the toxicity of dioxins, oxidative stress is considered as one of the important ones. In the hepatic tissues of laboratory animals, induction of reactive oxygen species production, lipid peroxidation, DNA damage and decreased membrane fluidity was noted after a single exposure of TCDD. A previous study found hepatic concentrations of MDA, one of the indicators of lipid peroxidation, to increase by around 5-fold after a 4-week administration period with a dose of 1 mg/kg HxCN. This study
provided elevated MDA concentrations in the liver after 0.1 and 0.3 mg/kg HxCN. Moreover, in the highest dose, TAS was also found to be diminished (around 21%). This indicates the presence of general oxidative stress, in which reactive oxygen species are elevated, increasing the chance of damage occurring to cell structures, nucleic acids, lipids, and proteins.\(^3\)6

Hematological disturbances, especially thrombocytopenia, are also often-noted in laboratory animals following exposure to TCDD.\(^1\)6 They have also been observed after 13-week exposure of rats to a mixture of different dioxin congeners, where lower mean PLTs were caused by hemorrhage or anemia.\(^3\)7 In the present study, lower PLTs were observed after administration with 0.1 and 0.3 mg/kg HxCN, respectively. By contrast, no signs of hemorrhage or anemia were noted in the thrombocytopenic rats. This may indicate that a different mechanism is responsible. Thrombocytopenia can occur from decreased platelet production, increased platelet consumption or sequestration from an enlarged spleen.\(^3\)8 A measurement of MPV may be useful to assess the cause of this phenomenon, as it is elevated during increased platelet turnover, allowing the release of younger platelets, but reduced during decreased platelet production.\(^3\)9 As a dose of 0.3 mg/kg HxCN caused significant thrombocytopenia with elevated MPV, the former seems to be probable, but more research should be done to elucidate the mechanism behind it.

Many chloroorganic chemicals are known to affect the heme biosynthesis pathway. Among them, polyhalogenated aromatics such as PCBs, HCB (hexachlorobenzene) or TCDD seem to be of the greatest concern due to their being classified as POPs: the most dangerous environmental poisons. PCNs were recently included into this group, and one of the aims of this study was to make a preliminary assessment of their influence on heme synthesis, it being one of the first to do so. TCDD is well known as a very potent inducer of porphyria in mice, even after single doses.\(^1\)7 In rats, hepatic porphyria is related only to chronic administration of TCDD and this effect was not found to occur after a single administration, even a high dose.\(^4\)0 It is also noteworthy that female rats are significantly more sensitive than male rats to the porphyrrogenic effect of halogenated aromatics.\(^1\)8 HxCN administered to rats for 90 days produced hepatic uroporphyria in as small a dose as 0.1 mg/kg. It could be assumed that this effect, like for other halogenated aromatics, is a result of inhibition of URO-D: its activity was found to be diminished in the present study, but this difference was statistically significant that is around 30%, only at the highest dose. The proposed mechanism of URO-D inhibition in rodents is through the expression of the gene associated with the Ah receptor, where TCDD acts as its ligand.\(^1\)7,\(^1\)8 It is possible that inhibition of this enzyme by HxCN can be also connected with AhR, as higher chlorinated PCNs cause rapid activation of this receptor.\(^4\)1 Moreover,
PCN67, the main component of HxCN, was proven to act through this receptor.\textsuperscript{42}

Data about the inhibition of ALA-D due to exposure to polyhalogenated aromatics is rather scarce. In rats, a technical mixture of PCBs (Aroclor 1254) was found to significantly reduce this enzyme activity by 50% in 48 h when injected subcutaneously in a single dose,\textsuperscript{43} whereas octabromodiphenyl ether diminished ALA-D activity by over 30% after repeated per os exposure.\textsuperscript{44} HxCNs also appear to cause the ALA-D inhibition: its activity was found to be significantly reduced compared to the control group after a dose of 0.3 mg/kg. The mechanism of this action remains unclear. Fujita et al. suggest that ALA-D activity could be inhibited by chemical modification of the active site of the enzyme, as demonstrated for trichloroethylene.\textsuperscript{45} Another mechanism could be action via restriction of ALA-D synthesis, which results in its concentration decreasing; this is believed to be the mode of action of styrene or bromobenzene.\textsuperscript{45}

An elevated level of ALA in urine is a concomitant effect of diminished ALA-D activity,\textsuperscript{46} which was also noted in our study. At the highest dose, an almost 3-fold increase in the urine level of ALA was accompanied with a more than 6-fold reduction in the concentration of lower carboxylated porphyrins, especially COPROs. These could result from the inhibition of both ALA-D and URO-D in the liver of female rats. As two steps of heme biosynthesis in this organ are disrupted, the disturbances in the urine described above could be attributed to the joint action of both the weakened formation of PBG and the decarboxylation of uroporphyrinogen.

It has been found that an increase in ALA-S is associated with the inhibition of heme synthesis. It is known, that regulation of this enzyme is controlled via negative feedback by heme.\textsuperscript{47} Mice fed with Kanechlor-500 (mixture of PCBs) demonstrated a decrease in the heme pool, which seemed to be caused by an inhibition of URO-D; this could be responsible for the remarkable induction of ALA-S.\textsuperscript{48} Moreover, exposure of female rats to TCDD for 16 weeks also resulted in the induction of ALA-S.\textsuperscript{40} Although diminished ALA-D and URO-D activity was observed in the present study, the activity of ALA-S seems to be unaffected by the HxCN, but a slight tendency to decrease in its activity may be noticed. As only one point of time was examined during the administration period in the present study, it is very difficult to state if any changes in the activity of ALA-S occurred over the time of exposure. However, reduced activity was also demonstrated after 21 days.

FIGURE 5 Porphyrins concentrations (% of Control) in the liver and urine of rats following subchronic HxCN administration. Control values for hepatic porphyrins: URO 94.15 ± 40.30 pmol/g tissue; HEPTA 16.65 ± 7.10 pmol/g tissue; HEXA 2.45 ± 0.24 pmol/g tissue; PENTA 1.55 ± 0.20 pmol/g tissue; COPRO 5.72 ± 0.97 pmol/g tissue; TOTAL (total porphyrin concentration) 120.54 ± 47.67 pmol/g tissue. Control values for urinary porphyrins: URO 65.47 ± 18.59 pmol/mL; HEPTA 17.55 ± 3.07 pmol/mL; HEXA below limit of detection; PENTA 7.56 ± 2.65 pmol/mL; COPRO 366.39 ± 156.13 pmol/mL; TOTAL (total porphyrin concentration) 456.97 ± 168.54 pmol/mL. All values expressed as means ± SD, URO and COPRO presented as sum of isomers I and III; a: Significantly different from the control group, \(P < .05\); b: significantly different from the 0.03 group, \(P < .05\); c: significantly different from the 0.1 group, \(P < .05\)

FIGURE 6 Concentrations of ALA in the urine following subchronic HxCN administration in female rats. All values expressed as means ± SD; a: Significantly different from the control group, \(P < .05\); b: significantly different from the 0.03 group, \(P < .05\)
of octabromodiphenyl ether administration to rats, with concomitant inhibition of ALA-D.44

It should be highlighted that over 50% of the hepatic heme biosynthesis is committed to the synthesis of P450 enzymes.47 Thus, disruption of this path may also affect the level of hepatic cytochromes. Administration with two lower doses of HxCN results in around 30% increase in hepatic cytochrome P450 level; whereas 0.3 mg/kg HxCN caused conversely a significant decrease of over 30%. The latter may be caused by the inhibition of both ALA-D and URO-D, which result in diminished heme content. Similar results were obtained with

FIGURE 7  Histopathological pictures of rat liver sections: A and C—liver of control rat; B—liver of rat exposed to 0.3 mg/kg b.w. HxCN showing vacuolization of hepatocytes; D—liver of rat exposed to 0.3 mg/kg b.w. HxCN showing macro- and microvesicular steatosis localized mainly in the centrolobular zone. HE, hematoxylin/eosin staining (magnification ×120); OR, oil red staining (magnification ×240) [Color figure can be viewed at wileyonlinelibrary.com]

FIGURE 8  Hematological parameters following subchronic HxCN administration in female rats. All values expressed as means ± SD; a: significantly different from the control group, \( P < .05 \); b: significantly different from the 0.03 group, \( P < .05 \); c: significantly different from the 0.1 group, \( P < .05 \)
trichloroethylene, where inhibition of ALA-D in rats was connected with reduced cytochrome P450 concentration.\(^{45}\) Although concentration of cytochrome P450 in rats administered with 0.3 mg/kg body weight was decreased, the activity of CYP1A1 was enormously elevated, by over a 100-fold, with this increase being noted with all used doses. HxCN were found to be potent inductors of CYP1A1 in acute and subacute studies. Significant, dose-dependent induction of hepatic CYP1A1 was noted after 2-week exposure of both PCN66 and PCN67 in a range of doses from 500 ng/kg to 500 µg/kg and 1500 ng/kg to 500 µg/kg, respectively.\(^{12}\) Repeated administration of HxCN (doses of 1 and 10 mg/kg) triggered significant induction of CYP1A1, regardless of the administration period (up to 4 weeks). Moreover, this activity was continuously elevated for 7 weeks following the termination of the 28-day exposure period.\(^{13}\)

Additionally, the results of the in vivo micronucleus test suggest that HxCN, like TCDD, is probably not a genotoxic agent in female rats. Previous studies have reported positive results in the case of TCDD, but only after very high doses, and the results suggest that this could be a secondary effect to other toxic effects.\(^{49}\)

In conclusion, similarly to the other halogenated aromatics, subchronic exposure to HxCN affects the heme biosynthesis pathway and leads to hematological disturbances. HxCN administered to rats for 90 days at the highest dose of 0.3 mg/kg is a potent inhibitor of two crucial steps in heme synthesis. Whereas the dose of 0.1 mg/kg results in accumulation of higher carboxylated porphyrins in the liver, increased elimination of ALA in the urine and significant thrombocytopenia, the highest dose of 0.3 mg/kg induced also hepatotoxicity, expressed as hepatomegaly and fatty degeneration of the liver. The most sensitive effect of HxCN in rats was very strong induction of hepatic CYP1A1 activity, which was observed after exposure to 0.03 mg/kg, without other pronounced symptoms of toxicity. These findings shed a new light on the toxicity of HxCN, exposure to which, alongside with other halogenated aromatics such as PCBs and dioxins, could lead to harmful effects in humans that is porphyria or liver damage.

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