Exposure to the Widely Used Fungicide Mancozeb Causes Thyroid Hormone Disruption in Rat Dams but No Behavioral Effects in the Offspring

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The widely used fungicide mancozeb has been shown to cause hypothyroxinemia and other adverse effects on the thyroid hormone system in adult experimental animals. In humans, hypothyroxinemia early in pregnancy is associated with adverse effects on the developing nervous system and can lead to impaired cognitive function and motor development in children. The aim of the present study was therefore to assess whether perinatal mancozeb exposure would cause developmental neurotoxicity in rats. Groups of 9–21 time-mated Wistar rats were dosed with 0, 50, 100, or 150 mg mancozeb/kg body weight (bw)/day by gavage from gestation day (GD) 7 to postnatal day (PND) 16, and total thyroxine (T4) levels were measured in dams during gestation. On PND 16, hormone levels and several organ weights were measured in the offspring, whereas motor activity, startle response, and cognitive function were assessed in the adult offspring. The dose of 150 mg/kg/day caused neurotoxicity in the pregnant dams and was therefore reduced to 100 mg/kg bw/day in mid study. T4 levels showed a dose-dependent and significant decrease in dams from all three dose groups on GD 15, whereas offspring T4 levels, thyroid weights, and histology were unaffected on PND 16. No effects on reproductive organ weights were seen, and no behavioral changes were observed. Taken together, these results indicate that in rats, moderate maternal hypothyroxinemia during gestation does not necessarily lead to hyperactivity or reduced special learning abilities in the offspring. Mancozeb exposure did, however, reduce T4 levels in dams and may therefore still be a potential contributor to thyroid disruption in humans and in result adversely affects the developing brain.

Key Words: mancozeb; developmental neurotoxicity; behavior; rats; thyroid-disrupting chemicals.

Even mild changes in human thyroidal function in early prenatal life can have severe consequences for a child’s neurological development (Haddow et al., 1999; Henrichs et al., 2010; Kooistra et al., 2006; Li et al., 2010; Pop et al., 1999, 2003). This knowledge has prompted much focus on the importance of thyroid-disrupting chemicals (TDCs) in relation to neurological development (Howdeshell, 2002; Porterfield, 2000; Zoeller, 2007; Zoeller and Crofton, 2000), as numerous chemicals have been shown to adversely affect thyroid function (Brucker-Davis, 1998; Hurley et al., 1998). One group of TDCs are the fungicides from the dithiocarbamate family, which are widely used for protection of fruits, vegetables, and field crops from fungal diseases (WHO, 1988). The main degradation product of many of the dithiocarbamates is ethylene thiourea (ETU), a compound that exerts various toxic effects in rats, including thyroid neoplasms, developmental toxicity, and teratogenicity (NTP, 1992). The present study investigated the dithiocarbamate mancozeb, which is the most commonly sold fungicide in, e.g., Denmark (Danish EPA, 2010), Norway (Nordby et al., 2005), and the United States (Acquavella et al., 2003).

Mancozeb has been shown to cause adverse health effects in both humans and experimental animals. In a recent American agricultural health study, mancozeb exposure was strongly associated with increased incidence of thyroid disease in female spouses of pesticide applicators (Goldner et al., 2010), and a Norwegian study has shown a moderate association between mancozeb exposure and neural tube defects in newborns from farmer families (Nordby et al., 2005). In most of the published literature on the toxicological effects of mancozeb in rats, adult animals have been exposed to doses between 500 and 1500 mg/kg/day for longer periods of time. In these studies, mancozeb exerts numerous effects related to the function of the thyroid gland, including decreases in serum thyroxine (T4) levels, thyroid peroxidase activity and iodine uptake, increased production of thyroid stimulating hormone (TSH) and thyroid weight, hyperplasia and hypotropy of follicular cells in the thyroid, as well as thyroid cancer (Hurley et al., 1998; JMPR, 1993; Kackar et al., 1997b; Trivedi et al., 1993; WHO, 1988). Besides the thyrotoxic effects, reproductive effects in rats have been observed. These include decreased...
Mancozeb is small and is mainly a concern for agricultural and rats, including dyspnea, salivation, diarrhea, and paralysis of the hind limbs, followed by death of the animals (Kackar et al., 1997a; Mahadevaswami et al., 2000). Furthermore, higher dose levels of mancozeb have caused general toxicity as well as neurotoxic effects in rats, including dyspnea, salivation, diarrhea, and paralysis of the hind limbs, followed by death of the animals (Kackar et al., 1997b; Trivedi et al., 1993; WHO, 1988).

In humans, risk of acute intoxication by high doses of mancozeb is small and is mainly a concern for agricultural and industrial workers, but the population at large can be exposed to mancozeb and other dithiocarbamates through residues in food (Rossi et al., 2006). Together with exposure from other TDCs, this could potentially contribute to disruption of the thyroid hormone system. In pregnant women, such a disruption could have severe consequences for the neural development of unborn children, as hypothyroxinemia in the first part of pregnancy is associated with adverse effects on cognitive function and motor development in infants, toddlers, and young children (Henrichs et al., 2010; Li et al., 2010; Pop et al., 1999, 2003). The aim of the present study was therefore to investigate if mancozeb exposure during the pre- and postnatal period would affect any reproductive, thyroid, or behavioral endpoints in rat offspring.

MATERIALS AND METHODS

Two separate mancozeb studies were performed. In both studies, time-mated Wistar rats (HanTac:WH, Taconic Europe, Ejby, Denmark) were gavaged once daily from gestation day (GD) 7 to postnatal day (PND) 16 (day of delivery excluded). The dams were treated at a constant volume of 2 ml/kg body weight (bw)/day, with individual doses based on the bw of the animal on the day of dosing. The first study was a range-finding study with 24 time-mated dams (n = 6) and the second a larger dose-response study with 88 time-mated dams (n = 22). The studies were performed under conditions approved by the Danish Agency for Protection of Experimental Animals and by the in-house Animal Welfare Committee. In both studies, the animals received a complete rodent diet (Altromin Standard Diet 1314) and acidified tap water ad libitum and were housed under standard conditions as described in Axelstad et al. (2008).

The vehicle used was corn oil (Sigma-Aldrich, Brandby, Denmark). Mancozeb (CAS no. 8018-01-7, Lot.no. 371-131c) was from VWR—Bie & Berntsen, Herlev, Denmark. The supplier did not have information about the compound’s purity, only that it was a technical quality. The mancozeb solutions were prepared for each of the three study blocks. No verification of dose compound’s purity, only that it was a technical quality. The mancozeb solutions were prepared for each of the three study blocks. No verification of dose concentrations was performed.

In the range-finding study, which was run in one block, the doses were 0, 200, 350, or 500 mg/kg bw/day. After a few days of dosing, severe weight loss and signs of neurotoxicity (paralysis of the hind limbs) were observed in animals from all mancozeb groups. Consequently, all doses were halved on GD 12. However, the observed toxic effects continued, so on GD 14 all animals from the two highest dose groups and two animals in the lowest dose group were sacrificed. Two dams from the control group were sacrificed on GD 14 for comparison. Before sacrifice, each dam was scored for severity of hind limb paralysis on a scale from 0 to 3. The remaining animals in the lowest dose group continued receiving 100 mg/kg bw/day from GD 14 to PND 16, at which point the study was terminated. This dose level did not cause further clinical signs of toxicity.

In the dose-response study, the plan was to dose the dams with 0, 50, 100, or 150 mg mancozeb/kg bw/day from GD 7 to PND 16. The study was divided into three blocks, with 1 week in-between, and an equal representation of each dose group in the blocks. After the first week of dosing, indications of toxic effects were observed in the highest dose group. Two of the animals suffered from severe weight loss and mild hind leg paralysis and were sacrificed on GD 16, and the highest mancozeb dose was reduced from 150 to 100 mg/kg bw/day. In consequence, the high-dose animals from the first block received 150 mg/kg from GD 7–21 and 100 mg/kg bw/day for the rest of the dosing period, whereas the animals in the second block only received 150 mg/kg bw/day from GD 7 to 14 and 100 mg/kg bw/day from GD 15 to PND 16. The animals from the first two blocks were nonetheless grouped when data analysis was performed. In the third block, dams from the high-dose group received 100 mg/kg during the whole treatment period and were therefore included as part of the middle dose group in the data analysis. Because of this alteration in dosing scheme, combined with a low pregnancy rate from the animal breeders, final group sizes differed considerably (Table 1).

The day after delivery, the pups were counted, sexed, weighed, checked for anomalies, and anogenital distance (AGD). Bw were measured again on PND 6, 13, 24, 31, and 45. Offspring were examined for the presence of nipples/areolas on PND 13 and for motor activity levels on PND 14, 17, and 23 (all procedures are further described in Axelstad et al., 2008). On PND 16, three male and two female pups per litter were sacrificed, when possible. They were weighed and decapitated, and testes, epididymis, ventral prostate, ovaries, liver, adrenals, and thyroid glands were excised, weighed, and prepared for histopathological examinations. On PND 24, 2–4 pups per litter were weaned and kept for assessment of developmental neurotoxicity, whereas the dams and one male per litter were sacrificed. In dams, the thyroids were excised, weighed, and prepared for histopathology and the number of implantation scars in the uterus was registered. In offspring, thyroid gland and testes were excised, weighed, and prepared for histopathology. All organs intended for histopathological examinations were fixed in formalin, embedded in paraffin, and stained with hematoxylin and eosin. Histological evaluations were made if statistically significant changes in organ weights were seen. Pup thyroids, testes, and ovaries on PND 16 were evaluated histologically, irrespectively of organ weight changes.

Several blood samples were collected for hormone analysis. On GD 15, dams were anesthetized with Hypnorm® (fentanyl citrate/flunisone) and blood was drawn from the tail vein. From offspring on PND 16 and PND 24 and from dams on PND 24, trunk blood was collected. Total T4 content in plasma was measured in all samples using modified DeltaT T4 kits (as described in Axelstad et al., 2008). Testosterone was extracted from male PND 16 serum as previously described (Vinggaard et al., 2005) and was measured by time-resolved fluorescence using commercially available fluorimuno- assay kits (PerkinElmer Life Sciences, Turku, Finland).

The adult offspring (3–7 months old) were tested in a battery of behavioral tests. Experimental setup for testing of spatial learning in a radial arm maze and motor activity in activity boxes is described in Axelstad et al. (2008). Testing for acoustic startle response was performed as described in Kjaer et al. (2010), with some modifications, by use of the SR-Lab TM SDI startle response system (SanDiego Instruments, Inc., Europe). Specifically, no background noise was detected in the two chambers during testing. After 5 min acclimatization, the test session commenced with five 120-dB(A) white noise startle trials for habituation followed by 100 test trials delivered in semirandomized order: 10 startle trials of 120 dB(A) white noise; 10 each of 8 prepauses (25, 30, 35, 40, 45, 50, 55, and 60 dB(A) white noise, respectively), and 10 trials with no stimuli. Movement of the tube was registered for 100 ms after onset of the startle stimulus and amplified, and the average response over 100 ms (AVG) was calculated. For each level of prepulse, AVGs were averaged and used for calculation of prepulse inhibition (PPI). PPI was expressed as percent reduction in AVG compared with the average of the 10 middle 120-dB startle trials: % PPI = 100 − [AVG at prepulse + startle trial]/(AVG at startle trial) 100%.

Animals were weighed before each behavioral test. At 8 months of age, all offspring were anesthetized with CO2/O2 and decapitated. Ovaries and thyroid glands were excised, weighed, and prepared for histopathology.
Statistical analysis of data with normal distribution and homogeneity of variance were analyzed using ANOVA followed by a Dunnett post hoc test as described in Axelstad et al. (2008). When more than one pup from each litter was examined, statistical analyses were adjusted using litter as an independent, random, and nested factor in ANOVA or analyses were done on litter means. In cases where normal distribution and homogeneity of variance could not be obtained by data transformation, a nonparametric Kruskall-Wallis test was used. Trend analysis on dose-response relations for hormone levels, body, and organ weights were performed using Spearman’s test. Statistical analyses of the effects on macroscopic lesions and histopathology were done using Fisher’s exact test.

**RESULTS**

The results from the two studies showed that doses of 150 mg/kg bw/day and above caused toxic effects in the dosed dams, as severe weight loss and transient paralysis of the hind limbs were observed. In the range-finding study, most dams were sacrificed on GD 14. At the time of necropsy, almost no food content was found in the ventricles and intestines of the sacrificed animals. The severity of hind limb paralysis was independent of pregnancy status but was dose dependent, as four out of six animals in the highest dose group received the most severe paralysis score, whereas two out of six animals in the middle dose group and none in the low-dose group received this score (data not shown). In the dams surviving after GD 14, maternal and pregnancy data, organ weights, and T4 concentrations were not analyzed because of the very small final group size in dams giving birth (n = 2–3).

In the main dose-response study, the two high-dose dams that were sacrificed on GD 16 received the mildest paralysis score. In the rest of the animals, maternal bw gain from GD 7 to GD 21 was significantly lower bw than controls (p < 0.001), and both endpoints showed a significant dose-dependent downward trend (p < 0.0001). During the lactation period, exposed dams generally gained more weight than controls, but the difference was only statistically significant in the high-dose group (p = 0.007). The additional weight gain was, however, not enough to eliminate differences in dam bw, and at the time of weaning (PND 24), high-dose dams had significantly lower bw than controls (p = 0.031) (Table 3), and trend analysis showed a dose-dependent decrease in dam bw
Gestation length, litter size, postimplantation loss, neonatal deaths, gender distribution, AGD, and nipple retention were similar in the four groups (Table 1). Offspring bw were significantly lowered in the high-dose group at birth \((p = 0.0004)\) and on PND 6 \((p = 0.036)\), with a significant dose-dependent downward trend on both days \((p = 0.0018\) and \(p = 0.0077\), respectively). From PND 13 to 45, offspring bw in the highest dose group seemed lower than in controls, and the trend analysis showed a significant downward trend on days 13, 24, and 31 \((p = 0.0154, p = 0.0185,\) and \(p = 0.0059\), respectively); however, the difference between groups was not statistically significant (Table 1). From PND 45 and onward, no effect on bw was seen (data not shown).

On GD 15, a significant dose-dependent downward trend was seen for \(T_4\) levels in dam serum \((p < 0.0001)\) and the levels were decreased by 21, 27, and 37% in the three mancozeb groups, respectively, compared with controls \((p = 0.0004, p < 0.0001,\) and \(p < 0.0001)\) (Fig. 1). This effect was no longer evident 1 week after dosing had stopped (PND 24) (Table 2), at which time also thyroid gland weights were unaffected (Table 3) and no dose-dependent trends were seen for any of the endpoints.

The offspring \(T_4\) levels on PND 16 showed no dose-dependent trends, and group means did not differ significantly from controls in any dose group (Table 2). On PND 24, no significant differences between groups were seen on offspring \(T_4\) levels (Table 2); however, a significant dose-dependent upward trend was seen \((p = 0.0242)\), indicating a possible reactive overshoot because of recovery from previously compromised thyroid hormone status. Thyroid gland weights in the offspring were unaffected on PND 16 as no dose-dependent trends and no significant differences between groups were seen (Table 3). Also no histopathological effects on the offspring thyroids on PND 16 were seen (data not shown). On PND 24 (Table 3) and in adulthood (data not shown), offspring thyroid weights were also unaffected and therefore histopathology was not investigated.

**TABLE 2**

<table>
<thead>
<tr>
<th>Hormone measurements (nM)</th>
<th>Control</th>
<th>50 mg Mz</th>
<th>100 mg Mz</th>
<th>150/100 mg Mz</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of samples</td>
<td>14–15</td>
<td>15–20</td>
<td>17–22</td>
<td>8–10</td>
</tr>
<tr>
<td>(T_4) in dams GD 15</td>
<td>41.4 ± 7.7</td>
<td>32.7 ± 6.3***</td>
<td>30.15 ± 6.7***</td>
<td>26.26 ± 5.2***</td>
</tr>
<tr>
<td>(T_4) in dams PND 24</td>
<td>24.8 ± 12.9</td>
<td>21.2 ± 7.9</td>
<td>19.6 ± 7.8</td>
<td>23.24 ± 8.6</td>
</tr>
<tr>
<td>(T_4) in offspring PND 16 (litter mean)</td>
<td>32.5 ± 7.5</td>
<td>35.8 ± 8.0</td>
<td>34.6 ± 5.4</td>
<td>33.1 ± 8.8</td>
</tr>
<tr>
<td>(T_4) in offspring PND 24 (litter mean)</td>
<td>18.5 ± 7.0</td>
<td>19.8 ± 7.6</td>
<td>22.1 ± 5.8</td>
<td>24.3 ± 6.2</td>
</tr>
<tr>
<td>Testosterone PND 16 (male)</td>
<td>1.05 ± 0.75</td>
<td>1.58 ± 1.70</td>
<td>1.54 ± 1.70</td>
<td>1.65 ± 1.40</td>
</tr>
</tbody>
</table>

**Note.** Mz, mancozeb.
TABLE 3
Effect of Mancozeb on Terminal Bw and Relative Organ Weights in Male and Female Rat Offspring Sacrificed at PND 16 and PND 24, after Exposure to 0, 50, 100, Or 150/100 mg Mancozeb/kg bw/day from GD 7 to PND 16. Data Represent Group Means Based on Litter Means ± SDs. Liver, Thyroid, and Adrenal Weights Are Litter Mean Values from Male and Female Offspring Combined. Asterisks Indicate a Statistically Significant Difference Compared with Controls *p < 0.05

<table>
<thead>
<tr>
<th>Organ weights PND 16</th>
<th>Control</th>
<th>50 mg Mz</th>
<th>100 mg Mz</th>
<th>150/100 mg Mz</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of litters</td>
<td>15</td>
<td>19</td>
<td>21</td>
<td>9</td>
</tr>
<tr>
<td>Bw (g)</td>
<td>30.60 ± 3.6</td>
<td>29.41 ± 6.4</td>
<td>28.06 ± 3.3</td>
<td>26.94 ± 2.0</td>
</tr>
<tr>
<td>Adrenal gland (mg/100 g bw)</td>
<td>33.59 ± 4.2</td>
<td>32.01 ± 5.1</td>
<td>32.14 ± 2.3</td>
<td>31.07 ± 2.5</td>
</tr>
<tr>
<td>Liver (mg/100 g bw)</td>
<td>2674 ± 116</td>
<td>2656 ± 63</td>
<td>2631 ± 117</td>
<td>2637 ± 130</td>
</tr>
<tr>
<td>Thyroid gland (mg/100 g bw)</td>
<td>16.36 ± 2.3</td>
<td>16.92 ± 2.4</td>
<td>16.83 ± 1.7</td>
<td>17.97 ± 1.7</td>
</tr>
<tr>
<td>Ovaries (mg/100 g bw)</td>
<td>43.39 ± 7.2</td>
<td>43.45 ± 7.3</td>
<td>44.85 ± 5.9</td>
<td>49.07 ± 5.2</td>
</tr>
<tr>
<td>Testes (mg/100 g bw)</td>
<td>353.3 ± 24.4</td>
<td>349.5 ± 24.5</td>
<td>350.5 ± 20.1</td>
<td>347.6 ± 11.8</td>
</tr>
<tr>
<td>Epididymis (mg/100 g bw)</td>
<td>97.94 ± 19.2</td>
<td>94.01 ± 22.6</td>
<td>88.06 ± 21.3</td>
<td>111.4 ± 26.2</td>
</tr>
<tr>
<td>Prostate (mg/100 g bw)</td>
<td>40.23 ± 11.6</td>
<td>44.83 ± 11.9</td>
<td>40.30 ± 6.5</td>
<td>46.91 ± 9.2</td>
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</table>

<table>
<thead>
<tr>
<th>Organ weights PND 24</th>
<th>Control</th>
<th>50 mg Mz</th>
<th>100 mg Mz</th>
<th>150/100 mg Mz</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dam bw (g)</td>
<td>254 ± 17</td>
<td>250 ± 24</td>
<td>247 ± 14</td>
<td>234 ± 13*</td>
</tr>
<tr>
<td>Dam thyroid (mg/100 g bw)</td>
<td>6.52 ± 0.72</td>
<td>6.12 ± 0.85</td>
<td>6.57 ± 1.04</td>
<td>6.32 ± 0.99</td>
</tr>
<tr>
<td>Bw in male offspring (g)</td>
<td>61.43 ± 5.7</td>
<td>57.50 ± 12.7</td>
<td>55.20 ± 3.5</td>
<td>55.00 ± 4.3</td>
</tr>
<tr>
<td>Thyroid gland (mg/100 g offspring)</td>
<td>13.04 ± 3.07</td>
<td>14.52 ± 3.31</td>
<td>13.18 ± 2.64</td>
<td>14.76 ± 5.74</td>
</tr>
<tr>
<td>Testes (mg/100 g bw)</td>
<td>569.9 ± 34.2</td>
<td>585.8 ± 71.96</td>
<td>573.5 ± 61.23</td>
<td>556.6 ± 54.64</td>
</tr>
</tbody>
</table>

Note. Mz, mancozeb.

On PND 16, weights of testes, epididymis, prostate, ovaries, and liver in the offspring were also unaffected by mancozeb exposure (Table 3). Statistical analysis of the relative organ weights and of absolute organ weights analyzed with bw as covariate indicated no differences between treatment groups, and no dose-dependent trends were seen. No histopathological effects were seen in the ovaries and testes of the offspring (data not shown), whereas histopathology of the other organs was not investigated. On PND 24, there were no effects on testes weights (Table 3), and in adult female offspring, there were no effects on ovary weights (data not shown).

None of the performed behavioral tests showed effects of mancozeb exposure, as neither activity levels in young or adult offspring, performance in the radial arm maze, or acoustic startle response were affected nor were any dose-dependent trends seen (data not shown).

DISCUSSION

The observed neurotoxic effects in the dams corresponded well with the toxicity effects generally seen after higher dithiocarbamate exposures (ataxia and paralysis of the hind limbs caused by demyelination and degeneration of peripheral nerve cells) (WHO, 1988); however, the dose levels were surprisingly low compared with doses causing adverse effects in other published studies. Dose-dependent weight loss and hind limb paralysis were observed in dams from all dose groups in the range-finding study. Furthermore, some of the animals dosed with 150 mg/kg bw/day in the dose-response study displayed clinical signs of intoxication and had to be sacrificed after 7 days of dosing.

In the published literature, similar toxic effects of mancozeb have been reported but at much higher dose levels (500–1500 mg/kg bw/day) and after longer dosing periods than seen in the present study (Kackar et al., 1997a,b; Trivedi et al., 1993). In these studies, mortality rates between 15 and 20% were seen in animals receiving 500 mg/kg bw/day for a year, whereas no signs of weight loss or any clinical signs of neurotoxicity were reported in female rats receiving between 500 and 800 mg mancozeb/kg bw/day for a month (Baligar and Kaliwal, 2001; Mahadevswami et al., 2000). In contrast to these published findings, a number of unpublished industry studies of mancozeb exist. The results from these are used for risk assessment purposes and are referred in reports from the Joint Meeting on Pesticide Residues (JMPR, 1993). In these studies, the mancozeb doses used were generally much lower than seen in the published mancozeb literature, and the no adverse effect levels (NOAELs) for mancozeb were between 5 and 10 mg/kg bw/day, whereas lowest adverse effect level (LOAELs) were between 10 and 50 mg/kg bw/day. These were based on a number of short- and long-term toxicity, reproductive, and teratogenicity studies in rats, and the critical effects were most often bw reductions and decreased food consumption but also organ weight changes, histopathological findings (often in the thyroid), and altered hormone levels of T4 and TSH (Gallo et al., 1980; Goldman et al., 1986; Hooks et al., 1992; Muller, 1992 in JMPR 1993; Stadler, 1990; Sundar, 1999; Tesh et al., 1988). Hind leg paralysis was reported in one study, where pregnant dams were dosed with 360 mg mancozeb/kg bw/day from GD 6 to 15 but was not seen in the 60 mg/kg bw/day dose (Tesh et al., 1988). Which other dose levels between 60 and 360 mg/kg/day would have caused this effect is speculative.
It is unclear why the dose levels inducing toxic effects in the published literature were so much higher than seen in the industry studies and in the present one. Many different rat strains have been used, and the animals have been dosed either by gavage or in the feed, but no consistency in sensitivity of specific strains or dosing method was evident from the data. A possible explanation could be the purity of the tested mancozeb. In all the unpublished studies, technical grade quality with purity between 82 and 86% was used. In the published literature, the mancozeb was often a commercial grade quality, with no stated purity, and it may therefore have differed from the technical grade quality either in the amount of mancozeb present or in the amount or types of impurities.

Mancozeb exposure reduced plasma T\textsubscript{4} levels in the dams in a dose-dependent manner, with the highest dose of 150/100 mg/kg bw/day causing a 37% decrease after 7 days of dosing. Because of the switch in dosing from 150 to 100 mg/kg bw/day, it was not possible to discern if effects seen in the highest dose group were caused by the short-term exposure to 150 mg mancozeb/kg bw/day or the overall dose produced by combined 150/100 mg/kg.

In some of the published literature, T\textsubscript{4} reductions around 50% were first seen after either 2 months or 1 year of dosing with 1500 mg mancozeb/kg bw/day (Kackar et al., 1997b; Trivedi et al., 1993). However, in a more recent study, mancozeb exposure induced reductions in T\textsubscript{4} levels comparable to those of the present study, as a 50% reduction in T\textsubscript{4} levels was seen after 4 days of dosing with 250 mg mancozeb/kg bw/day. Effects on body weight and other general toxicity effects were not seen in this study, even at quite high-dose levels (250, 500, and 1000 mg/kg/day), but this was probably because of the relatively short dosing period of 4 days (Flippin et al., 2009). In the mancozeb studies referred in the JMPR report, LOAELs for T\textsubscript{4} reductions in two long-term toxicity studies were between 450 and 750 ppm mancozeb in the diet (equal to approximately 20–30 mg/kg bw/day) (Hooks et al., 1992; Stadler, 1990), which supports our findings of effects in the dams at 50 mg/kg bw/day.

In contrast to the many studies reporting increased thyroid weights (Hurley et al., 1998; JMPR, 1993; Kackar et al., 1997b; Trivedi et al., 1993; WHO, 1988), no effect on thyroid weight was seen in dams from the present study when measured on PND 24. However, at this time, dosing had already been discontinued for 7 days, and an effect on the maternal thyroid might therefore have been reversed.

Interestingly, offspring levels of T\textsubscript{4} were not significantly lowered in any dose group compared with controls when measured on PND 16. It is, however, unclear whether the thyroid hormone system of the offspring was unaffected by mancozeb during the entire dosing period. The fact that a significant dose-dependent upward trend was seen in offspring T\textsubscript{4} levels on PND 24 may indicate a possible reactive overshoot because of recovery from previously compromised thyroid hormone status. Therefore, measurements of T\textsubscript{3} levels during fetal or early postnatal life might have revealed other results than seen on PND 16. Such a pattern in postnatal T\textsubscript{4} levels can be seen in a recent publication from Paul et al. (2010). Here the TDC tricoslan was given to rat dams during gestation and lactation, and whereas offspring T\textsubscript{4} levels were reduced by 30% on PND 4, no significant effects were seen on PND 14 and PND 21. According to the literature, the thiocarbamates and their metabolite ETU can cross the placenta and are found in breast milk (Brucker-Davis, 1998). It is, however, possible that the mancozeb did not cross the placenta or pass to the milk in sufficient amounts to affect the thyroid status of the offspring. Possibly, toxicokinetic factors may have affected maternal transfer of mancozeb into milk and thereby limited lactation exposure to the pups. Alternatively, mancozeb may not have triggered the same toxicodynamic effects in the offspring during the lactation period as in exposed dams. Measurements of maternal and offspring T\textsubscript{4} levels during the early postnatal period would have been very helpful in discerning whether dams were more susceptible to the thyroid-disrupting effect of mancozeb than the offspring and for identifying whether the offspring’s thyroid system was affected at any time point during the dosing period. In future studies, measurements of mancozeb and its metabolites in milk, urine, or blood samples from the offspring would be helpful in order to learn more about transfer of mancozeb from dams to the offspring and to compare the exposure to human levels.

Developmental hypothyroidism in rats is known to reduce growth from the age of approximately 2–3 weeks and onward (Akaite et al., 1991; Kobayashi et al., 2005; Noda et al., 2005). In the present study, high-dose offspring were only significantly smaller than controls at birth and on PND 6; however, significant dose-dependent trends were seen on days 13, 24, and 31, and it is therefore unclear whether the weight reduction in the offspring was a consequence of developmental hypothyroxinemia or a direct effect of mancozeb, possibly related to the lower weight gain seen in the high-dose dams.

AGD, nipple retention, testosterone levels, reproductive organ weights, and histology on PND 16 were not affected by mancozeb, indicating that the perinatal exposure did not affect reproductive development. Previously, adverse effects on the reproductive system have been reported in the published literature, as treatment of adult male rats with mancozeb has caused adverse effects on testes and epididymis weights and histology (Kackar et al., 1997a). However, the effects were only seen after long-term exposure (180–360 days) to very high doses of mancozeb (1000–1500 mg/kg bw/day). Treatment of adult female Wistar rats with 500, 600, 700, or 800 mg mancozeb/kg bw/day for 15 and 30 days, respectively, affected the female reproductive system in the two highest dose groups. Effects included decreased ovarian weight, number of healthy follicles and estrous cycles, prolonged diestrus phase, and increased number of atretic follicles (Baligar and Kaliwal, 2001; Mahadevaswami et al., 2000). However, in these studies, no general toxicity effects were seen, which makes it difficult to compare the dose levels directly to the present ones. In the reproductive and developmental studies
referred in the JMPR, NOELs for reproduction were set much higher than for general toxicity effects, e.g., body changes and adverse effects on the thyroid hormone system. As described earlier, because of toxicokinetic and toxicodynamic effects, the mancozeb may not have passed to the offspring in sufficiently high amounts to affect their reproductive development. Furthermore, many of the endpoints affected in previous studies like estrous cyclicity, weight, and histology of reproductive organs in adult animals (that were still being dosed) were not investigated in the present study.

None of the investigated behaviors were affected by mancozeb exposure. Based on a large body of published literature on this subject, as well as on a developmental neurotoxicity study of the thyrotoxic compound propyl thiouracil from our own group, we had expected developmental hypothyroxinemia to cause elevated activity levels and impaired maze performances in the adult rat offspring (Akaike et al., 1991; Axelstad et al., 2008; Goldey et al., 1995; Kobayashi et al., 2005; Noda et al., 2005). Furthermore, we have previously hypothesized that the degree of pre- and postnatal hypothyroxinemia could be directly correlated to changes in these behaviors (Axelstad et al., 2008), in a similar way to what was proposed by Crofton (2004) for hearing ability. This hypothesis was, however, not corroborated in a later study from our group (Axelstad et al., 2011). Here exposure to the UV-filter octyl methoxycinnamate during gestation and lactation caused severe hypothyroxinemia in dosed dams, during both gestation and lactation, but only small effects on offspring T4 levels on PND 16 and no hyperactivity or impaired maze learning (Axelstad et al., 2011). We therefore hypothesized that in rats, T4 levels in the offspring needed to be severely reduced postnatally for effects on activity and learning abilities to appear. The results from the present study corroborate this hypothesis, as moderate maternal hypothyroxinemia during early gestation did not affect any of the investigated behaviors in the offspring.

There are, however, other studies which have shown effects on neural development in rat offspring after short-term maternal hypothyroxinemia (Auso et al., 2004; Opazo et al., 2008), so more research in this area is needed in order to elaborate if maternal hypothyroxinemia during gestation in rats is enough to adversely affect the behavior of the developing offspring.

It is, however, quite clear that in humans maternal T4 levels during the first trimester are crucial for fetal brain development, and even slight maternal hypothyroxinemia can result in adverse effects on the child’s cognitive and motor function (Henrichs et al., 2010; Kooistra et al., 2006; Li et al., 2010; Pop et al., 1999, 2003). This means that even though developmental mancozeb exposure in the present study did not affect the investigated behaviors in the rat offspring, this should not lead to the conclusion that in humans mancozeb exposure is without risk for pregnant women and their fetuses. In the present study, 8 days of mancozeb exposure was enough to reduce T4 levels significantly in all dose groups. The LOAEL of 50 mg/kg bw/day for T4 decreases was much lower than previously reported in the published literature but corroborated the effects seen in industry studies quite well. Because humans are exposed to a variety of thyroid disrupters, all probably acting in a dose-additive manner (Flippin et al., 2009), mancozeb residues in food should be carefully monitored in order to protect pregnant women and their children from excessive exposure to TDCs.

In summary, the present study investigated the effects of developmental mancozeb exposure on thyroid hormone levels, reproductive, and neurological development of the offspring. We have shown that mancozeb exposure induced neurotoxicity in dams at and above 150 mg/kg bw/day. The observed changes in maternal T4 levels did not cause any behavioral effects in the offspring, and we therefore hypothesize that in rats, moderate prenatal T4 decreases in dams are not determining for adverse development of learning and motor skills in the offspring. However, 8 days of mancozeb exposure was enough to cause between 20 and 40% decreases in T4 levels in rat dams, which indicates that mancozeb exposure may be a potential contributor to thyroid disruption in humans.

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