Evaluation of Endocrine Disrupting Effects of Nitrate after In Utero Exposure in Rats and of Nitrate and Nitrite in the H295R and T-Screen Assay

Pernille Reimer Hansen, Camilla Taxvig, Sofie Christiansen, Marta Axelstad, Julie Boberg, Maria Kristina Kiersgaard, Christine Nelleman, and Ulla Hass

National Food Institute, Technical University of Denmark, Department of Toxicology and Risk Assessment, Mørkhøj Bygade 19, DK-2860 Søborg, Denmark

Received November 21, 2008; accepted January 28, 2009

Animal studies have shown that nitrate acts as an endocrine disrupter affecting the androgen production in adult males. This raises a concern for more severe endocrine disrupting effects after exposure during the sensitive period of prenatal male sexual development. As there are no existing studies of effects of nitrate on male sexual development, the aim of the study was to examine how in utero exposure to nitrate would affect male rat fetuses. Pregnant dams were dosed with nitrate in the drinking water from gestational day (GD) 7 to GD21 at the following dose levels 17.5, 50, 150, 450, and 900 mg/l. At GD21, fetuses were examined for anogenital distance, plasma thyroxine levels, testicular and plasma levels of testosterone and progesterone, and testicular testosterone production and histopathology. In addition, endocrine disrupting activity of nitrate and nitrite were studied in two in vitro assays, the H295R assay and T-screen. There were no consistent indications that nitrate induces anti-androgenic effects in male fetuses or that prenatal nitrate exposure affected the thyroid axis. However, a more comprehensive study with long-term exposure before and during pre- and postnatal development would be relevant to sufficiently address the concerns based on the indications for endocrine disrupting effects in adult animals.

Key Words: nitrate; nitrite; endocrine disruption; H295R assay; T-Screen; steroidogenesis; developmental toxicity; rat.

Humans are exposed to nitrate and nitrite through food and drinking water, with a minor contribution from the air (WHO, 2007). Nitrate is a salt of nitric acid, with an ion composed of one nitrogen and three oxygen atoms (NO$_3^-$), and the sources of nitrate in the drinking water are primarily from agricultural activities. Nitrite (NO$_2^-$, a salt of nitrous acid) can be formed in nitrate-containing and oxygen-poor water within galvanized steel pipes in the water distribution systems (WHO, 2007). The first health effects of nitrate were reported by Comly in 1945 after observing cyanosis in infants in Iowa (Comly, 1945). Since then there has been concern of possible health effects related to high nitrate consumption, including some forms of cancer (Foreman, 1989; Gulis et al., 2002), thyroid disorders (Tajtková et al., 2006; van Maanen et al., 1994) and reproductive effects (Bukowski et al., 2001; Manassaram et al., 2006; Ward et al., 2005).

The World Health Organization has set the limit values for nitrate and nitrite in drinking water to 50 and 3 mg/l, respectively. These values are based on epidemiological evidence for methemoglobinemia (WHO, 2007). In Europe the limit values in drinking water are 50 mg/l for nitrate and 0.5 mg/l for nitrite (EU, 1998). A report prepared by the European Environment Agency in 2003 stated that European public water supplies are largely below the limit for nitrate. Nevertheless in some countries, private wells in rural areas have elevated nitrate concentrations above 50 mg/l. Overall; nitrate levels exceeded 50 mg/l in about one-third of the groundwater bodies in Europe for which data were available (European Environment Agency, 2003).

Results from animal studies of both nitrite and nitrate exposure via drinking water have shown effects on male rat testosterone production and testicular size and for nitrate these effects have been seen at concentrations close to the allowed limit in drinking water (Panesar and Chan, 2000). Pant and Srivastava (2002) have reported effects of nitrate exposure on semen quality in male mice at 900 ppm. Effects on thyroid hormones and increased weight of the thyroid gland have been observed in adult female rats after nitrate exposure of 50 mg/l (Esciocak et al., 2005). Zaki et al. (2004) exposed male rats to nitrate in the drinking water and reported a dose-dependent increase in weight of the thyroid gland as well as decreased levels of triiodothyronine (T$_3$) at nitrate concentrations of 150 and 500 mg/l and a decrease in thyroxine (T$_4$) at 500 mg/l. Furthermore, increased thyroid volume has been reported in humans living in areas with high concentrations of nitrate in their drinking water. However, other possible thyroid disruptors than nitrate were not measured in the drinking water in these studies (Tajtková et al., 2006; van Maanen et al., 1994).

The indications for endocrine disrupting effects of nitrate and nitrite and especially their ability to interfere with androgen...
production in males raise a concern for effects during male sexual development. The prenatal period has been shown to be the most sensitive period for effects of numerous endocrine disrupting chemicals, including certain anti-androgenic phthalates. These phthalates have been shown to decrease the anogenital distance (AGD) and impair steroidogenesis in fetal and neonatal male rats at critical periods of sexual development leading to malformations in the male reproductive system and reduced sperm count later in life (Barlow et al., 2004; Borch et al., 2004, 2006; Carruthers and Foster, 2005; Ema et al., 2003, 2000; Gray et al., 2000; Parks et al., 2000). If nitrate and nitrite impair testosterone production also in fetal testes, effects comparable to those seen following phthalate exposure may be anticipated.

However, there are no studies of the effects of nitrate or nitrite on male sexual development. In order to investigate this, the most comprehensive study would be a two-generation study (OECD 416) or the currently discussed extended one-generation study (OECD 416) or the currently discussed extended one-generation study (OECD 416) and the two extracts were pooled and evaporated. Before analyzing, the heptane-fraction was transferred to a clean vial, the procedure was repeated, and the two extracts were pooled and evaporated. Before analyzing, the samples were resuspended in 100 μl of Dulbecco's modified Eagle's medium (DMEM)/F12; L-glutamin) medium. The plasma level of the thyroid hormone thyroxin (T4) in dams and female fetuses were analyzed by a skilled technician who has many years of experience in measuring AGD on GD 21 and PND1 pups. This was done as previously described in Borch et al. (2006) and Taxvig et al. (2008a). Fetuses were decapitated and trunk blood was collected into heparin-coated vials from dams and all fetuses for hormone analysis; one pool per litter was made for male and female fetuses, respectively. Fetal adrenals and testes were excised and sampled for hormone analyses and/or histopathology. Thyroid glands from dams were excised, weighed, and used for histopathological investigation.

Hormone Levels

Testosterone levels were analyzed in serum from male fetuses, as were ex vivo testicular testosterone productions as described in Laier et al. (2006).

After extraction of hormones from tissues with heparin, testosterone and progesterone levels were analyzed in testis extracts, and cortisol levels were measured in adrenal glands extracts. Testes and adrenal glands were placed in vials containing 100 μl of water and 0.5 ml of heparin. The tissues were homogenized and the vials placed in a test tube consisting of dry ice and acetone until the water-fraction was frozen. The heptane-fraction was transferred to a clean vial, the procedure was repeated, and the two extracts were pooled and evaporated. Before analyzing, the samples were resuspended in 100 μl of Dulbecco's modified Eagle's medium (DMEM)/F12; L-glutamin) medium. The plasma level of the thyroid hormone thyroxin (T4) in dams and female fetuses were analyzed by a skilled technician who has many years of experience in measuring AGD on GD 21 and PND1 pups. This was done as previously described in Borch et al. (2006) and Taxvig et al. (2008a). Fetuses were decapitated and trunk blood was collected into heparin-coated vials from dams and all fetuses for hormone analysis; one pool per litter was made for male and female fetuses, respectively. Fetal adrenals and testes were excised and sampled for hormone analyses and/or histopathology. Thyroid glands from dams were excised, weighed, and used for histopathological investigation.

Materials and Methods

Test Compounds

In the in vivo part of the study Sodium nitrate 99% (CAS no. 7631-99-4) from VWR & Bie & Berntsen was used. Sodium nitrate 99% (CAS no. 7632-00-0) and sodium nitrate 99% (CAS no. 7631-99-4) both from Sigma-Aldrich were used in the in vitro part of the study.

Animals and Exposure

Sixty-four time-mated, young adult Wistar rats (HanTac: WH, Taconic Europe, Ejby, Denmark) were supplied day 3 after mating. The animals were, upon arrival, randomly distributed in pairs and housed under standard conditions: semitransparent plastic-cages (15 × 27 × 43 cm) with Aspen bedding (Tapvei). They were housed in a room at a temperature of 22 ± 1°C and relative humidity of 55 ± 5%. The room was illuminated to give a cycle of 12-h light (9 P.M. to 9 A.M.) and 12 h of darkness (9 A.M. to 9 P.M.) as previously described in Vinggaard et al. (2005). The animals were fed soy and alfalfa free Altromin Diet 1314 ad libitum (Altromin GmbH, Lage, Germany). The day after arrival, that is, GD4, animals were weighed and assigned to six groups with similar weight distributions. An acclimatization period of 4 days was allowed before starting exposure. The study was run in three blocks, with one week in between each block and representation of each dose group in each block. The control group (n = 12) received filtered water and the five treatment groups received filtered water containing 17.5 mg/l (n = 12), 50 mg/l (n = 12), 150 mg/l (n = 12), 450 mg/l (n = 8), and 900 mg/l (n = 8) nitrate, respectively, from GD7 to GD21. Nitrate-water stocks were prepared weekly and two times per week water consumption was recorded and water replaced.

Health Status of Dams

The dams were observed daily for signs of toxicity. Body weights were recorded on GD4, GD7, GD10, GD14, GD17, and GD21 and the maternal weight gain from GD7 to GD21 was calculated.

Caesarean Sections GD 21

At GD21 the dams were weighed and decapitated after CO2/O2 anesthesia. Uteri were taken out, and the numbers of live fetuses, resorptions, and implantations were registered as previously described in Vinggaard et al. (2005). Body weights of the fetuses were recorded using a scale and AGD was measured in all fetuses using a stereomicroscope with an ocular reticle. The measurements were performed blinded with respect to treatment group by a skilled technician who has many years of experience in measuring AGD on GD21 and PND1 pups. This was done as previously described in Borch et al. (2006) and Taxvig et al. (2008a). Fetuses were decapitated and trunk blood was collected into heparin-coated vials from dams and all fetuses for hormone analysis; one pool per litter was made for male and female fetuses, respectively. Fetal adrenals and testes were excised and sampled for hormone analyses and/or histopathology. Thyroid glands from dams were excised, weighed, and used for histopathological investigation.
After incubation vials were centrifuged at 4000 × g for 10 min and the supernatants were stored at −80°C until cortisol levels were analyzed using the HitHunter Cortisol Assay Kit (Discoverx, Amersham Biosciences).

**Histopathology and Immunohistochemistry**

Testes from male offspring were fixed in Bouin’s fixative and embedded in paraffin. The testes were stained with hematoxylin and eosin for standard morphologic examination by light microscopy. Testes were examined for presence of multinuclear gonocytes, presence of Leydig cell clusters, Sertoli cell vacuolization and enlarged tubules with centrally located gonocytes. Furthermore the immunohistochemical staining for cytochrome P450 side chain cleavage (P450scC) was performed on testis sections as described in Borch et al. (2006).

Thyroid glands from dams were fixed in formalin, embedded in paraffin and examined by light microscopy after staining with hematoxylin and eosin. An observer blinded to treatment group evaluated all hematoxylin and eosin stained sections. The thyroids were examined for hyperplasia, changes in cell volume and cell height and changes in follicular volume. To determine gross hypothyroid, the thyroid glands were weighed.

**Cell Culture**

The T-Screen assay. The rat pituitary cell line GH3 obtained from American Type Culture Collection, ATCC, were cultured in a humid atmosphere at 37°C and 95% air/5% CO2 in phenol-red free Dulbecco’s modified eagle’s medium (DMEM/F12) (Gibco-Invitrogen, Paisley, UK) supplemented with 1% Antibiotic/Antimycotic (PSF) and 10% (vol/vol) fetal calf serum (FCS) (Gibco, UK). For the thyroid hormone-responsive cell proliferation assay (T-screen), cells were grown in test medium containing 10% (vol/vol) T3-depleted dextran-charcoal treated FCS (DC-FCS). Removal of thyroid hormone from DC-FCS was tested both in the absence or presence of 0.1nM T3 (T3-EC 50) to test for the presence of multinucleate gonocytes, presence of Leydig cell clusters, Sertoli cell vacuolization and enlarged tubules with centrally located gonocytes. Furthermore the immunohistochemical staining for cytochrome P450 side chain cleavage (P450scC) was performed on testis sections as described in Borch et al. (2006).

Thyroid glands from dams were fixed in formalin, embedded in paraffin and examined by light microscopy after staining with hematoxylin and eosin. An observer blinded to treatment group evaluated all hematoxylin and eosin stained sections. The thyroids were examined for hyperplasia, changes in cell volume and cell height and changes in follicular volume. To determine gross hypothyroid, the thyroid glands were weighed.

**Results**

**Doses and Water Consumption**

Two times per week water consumption of the dams was recorded and there were no difference in water uptake in control and treated animals (data not shown). The dose rates in mg nitrate/kg bw/day were calculated based on water intake and body weight in the different groups: 0 mg/kg/day (control), 2.0–3.0 mg/kg/day (17.5 mg/l), 6.4–8.6 mg/kg/day (50 mg/l), 18–23 mg/kg/day (150 mg/l), 60–75 mg/kg/day (450 mg/l), and 117–136 mg/kg/day (900 mg/l).

**In Vivo Effects**

**Pregnancy and litter data.** Nitrate exposure had no statistically significant effects on maternal body weight gain GD7–21, number of fetuses, postimplantation loss, AGD, or fetal weight (Table 1). No signs of toxic effects on the dams were observed during the exposure period.

**Hormone levels.** Plasma levels of total T4 in the dams at GD21 did not differ among groups (data not shown). In female fetuses, total T4 was under the detection limit in all samples measured except in three out of six samples in the highest exposure group. Therefore, total T4 was significantly increased...
in the highest dose group compared with control. However, because it was only possible to measure T4 in three samples in total, the result is questionable. In the male fetuses there were no statistically significant effects of the nitrate exposure on plasma levels of testosterone (Table 2). Testicular testosterone and progesterone levels, as well as ex vivo testicular testosterone production in male fetuses did not differ among groups either (Table 3).

Cortisol levels in adrenal glands from male fetuses as well as cortisol production measured in supernatant fluid from incubated adrenal glands from both male and female fetuses were similar across groups (Table 2).

**Histopathology and immunohistochemistry.** Testes from nitrate exposed fetuses and thyroid glands from exposed dams were histologically similar to controls. There were no significant differences in the weights of the thyroid glands of the dams, and no signs of hyperplasia, changes in cell volume and cell height or changes in follicular volume (data not shown). In testis sections, there were no treatment-related differences in the testes when examined for presence of multinucleate gonocytes, presence of Leydig cell clusters, Sertoli cell vacuolization, or enlarged tubules with centrally located gonocytes. Furthermore, no differences were observed in the intensity of immunostaining for P450scc (data not shown).

### Table 1

<table>
<thead>
<tr>
<th>Pregnancy and Litter Data</th>
<th>Control</th>
<th>17.5 mg/l NO3</th>
<th>50 mg/l NO3</th>
<th>150 mg/l NO3</th>
<th>450 mg/l NO3</th>
<th>900 mg/l NO3</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of dams</td>
<td>9</td>
<td>5</td>
<td>10</td>
<td>9</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>Maternal bw</td>
<td>305.4 ± 19.6</td>
<td>300.6 ± 9.8</td>
<td>303.2 ± 29.25</td>
<td>310.4 ± 28.4</td>
<td>313.7 ± 27.3</td>
<td>303.6 ± 27.3</td>
</tr>
<tr>
<td>Adjusted bw</td>
<td>239.4 ± 17.2</td>
<td>232.5 ± 7.7</td>
<td>238.6 ± 20.6</td>
<td>248.7 ± 11</td>
<td>249.7 ± 14.7</td>
<td>244.6 ± 24.1</td>
</tr>
<tr>
<td>No. of implantations</td>
<td>12.7 ± 1.3</td>
<td>12.6 ± 0.5</td>
<td>12.4 ± 3.2</td>
<td>11.8 ± 4.4</td>
<td>11.7 ± 3.2</td>
<td>11.0 ± 2.2</td>
</tr>
<tr>
<td>No. of fetuses</td>
<td>12 ± 1.5</td>
<td>12.2 ± 1.3</td>
<td>12.1 ± 3.4</td>
<td>11.2 ± 4.6</td>
<td>11.2 ± 3.3</td>
<td>10.3 ± 2.9</td>
</tr>
<tr>
<td>% postimplantation loss</td>
<td>5.3 ± 6.7</td>
<td>3.3 ± 7.5</td>
<td>3.7 ± 6.7</td>
<td>9.4 ± 15.9</td>
<td>5.2 ± 6.6</td>
<td>7.6 ± 10.5</td>
</tr>
<tr>
<td>Male fetal weight</td>
<td>3.56 ± 0.31</td>
<td>3.59 ± 0.12</td>
<td>3.53 ± 0.38</td>
<td>3.76 ± 0.80</td>
<td>3.66 ± 0.41</td>
<td>3.83 ± 0.72</td>
</tr>
<tr>
<td>Female fetal weight</td>
<td>3.41 ± 0.20</td>
<td>3.49 ± 0.19</td>
<td>3.40 ± 0.46</td>
<td>3.38 ± 0.18</td>
<td>3.47 ± 0.43</td>
<td>3.55 ± 0.63</td>
</tr>
<tr>
<td>Male AGD (mm)</td>
<td>3.58 ± 0.15</td>
<td>3.49 ± 0.13</td>
<td>3.50 ± 0.15</td>
<td>3.47 ± 0.17</td>
<td>3.49 ± 0.16</td>
<td>3.68 ± 0.17</td>
</tr>
<tr>
<td>Male AGD Index</td>
<td>2.35 ± 0.14</td>
<td>2.28 ± 0.10</td>
<td>2.31 ± 0.12</td>
<td>2.25 ± 0.19</td>
<td>2.27 ± 0.04</td>
<td>2.36 ± 0.12</td>
</tr>
<tr>
<td>Female AGD (mm)</td>
<td>1.98 ± 0.13</td>
<td>1.84 ± 0.07</td>
<td>1.94 ± 0.12</td>
<td>1.90 ± 0.11</td>
<td>1.91 ± 0.08</td>
<td>1.92 ± 0.18</td>
</tr>
<tr>
<td>Female AGD Index</td>
<td>1.32 ± 0.10</td>
<td>1.22 ± 0.06</td>
<td>1.29 ± 0.10</td>
<td>1.27 ± 0.09</td>
<td>1.27 ± 0.10</td>
<td>1.26 ± 0.15</td>
</tr>
</tbody>
</table>

**Note.** Bw, body weight. AGD was analyzed both with and without the cubic root of bw as a covariate, and both analyses showed that no groups were significantly affected. AGD Index means the AGD divided by the cubic root of body weight. Fetal body weight was analyzed using the live number of fetuses as a covariate. Data represent group means, based on litter means ± SD.

### Table 2

<table>
<thead>
<tr>
<th>Hormone Levels in Fetuses at GD 21</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (ng/adrenal gl.)</td>
<td>Mean (ng/adrenal gl.)</td>
</tr>
<tr>
<td>Control</td>
<td>4.41 ± 0.53 (9)</td>
<td>0.40 ± 0.18 (9)</td>
</tr>
<tr>
<td>17.5 mg/l NO3</td>
<td>4.31 ± 0.60 (5)</td>
<td>0.33 ± 0.08 (5)</td>
</tr>
<tr>
<td>50 mg/l NO3</td>
<td>4.19 ± 0.93 (10)</td>
<td>0.37 ± 0.10 (10)</td>
</tr>
<tr>
<td>150 mg/l NO3</td>
<td>4.13 ± 0.86 (8)</td>
<td>0.32 ± 0.03 (9)</td>
</tr>
<tr>
<td>450 mg/l NO3</td>
<td>3.69 ± 0.78 (6)</td>
<td>0.37 ± 0.06 (6)</td>
</tr>
<tr>
<td>900 mg/l NO3</td>
<td>4.74 ± 1.01 (7)</td>
<td>0.36 ± 0.07 (7)</td>
</tr>
</tbody>
</table>

**Note.** In male fetuses the plasma levels of testosterone at GD 21 were measured as described in “Materials and methods.” Cortisol levels were measured in adrenal glands in male fetuses and in supernatant fluid (cortisol production) from adrenal glands of both male and female fetuses. Regarding the T4 measurements most of them were below detection limit. Data represent group means ± SD; (t) = n; UD = under detection limit (0.2 nM); *statistically significantly different from control (p < 0.05).
or in combination with T₃, and consequently did not seem to act as neither thyroid receptor agonists nor antagonists.

**H295R steroid synthesis assay.** Neither sodium nitrate nor sodium nitrite had any dose-related effects on the production of testosterone or estradiol in H295R cells (Fig. 2). However, a significant decrease in testosterone production was seen for both compounds at 5 µM. Sodium chloride was included as a test of the effect of the sodium ion on the production of sex hormones. Even a concentration of 500 µM of NaCl had no effect in the H295R assay.

**DISCUSSION**

In this study, the main objective was to investigate if prenatal exposure to nitrate may induce anti-androgenic effects in male fetuses in a set-up that have been used with success to the detection of some anti-androgenic phthalates (Borch et al., 2004, 2006).

The results of the present study revealed no effects of treatment with nitrate up to 900 mg/l (117–136 mg/kg/day) on any of the measured hormone levels, or testicular testosterone production *ex vivo*. Previous studies have shown effects on male rat testosterone production and testes weight after 4 weeks of exposure to 50 mg/l of sodium nitrite or sodium nitrate in the drinking water (Panesar and Chan, 2000). Pant and Srivastava (2002) also reported effects on histopathology of the testes in adult male mice after exposure to 900 ppm potassium nitrate via drinking water for 35 days. However, no histopathological changes were observed on the fetal testis in the dosed groups compared with controls in the current study. Due to the suspected influence of nitrate on the steroid synthesis it was found relevant to include measurements on the level of cortisol. As for testosterone, no effects of nitrate exposure were observed on the level of cortisol in the given animal set-up.

AGD is a commonly used measure of demasculinization of rats exposed to anti-androgenic chemicals and effects on AGD have been observed in males exposed to e.g. certain phthalates *in utero* (Barlow et al., 2004; Borch et al., 2006; Carruthers and Foster, 2005; Ema et al., 2000, 2003). In the present study, no treatment-related effects on AGD were observed.

The lack of observed effects on steroidogenesis, histopathology of the testis and AGD may be due to the fact that the male fetuses in our study were exposed *in utero* and not directly in drinking water. Although some animal studies have

### TABLE 3
**Testicular Hormone Levels in Male Fetuses**

<table>
<thead>
<tr>
<th></th>
<th>Testosterone (ng/testis)</th>
<th>Testosterone production (ng/testis)</th>
<th>Progesterone (ng/testis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.97 ± 0.55 (9)</td>
<td>1.89 ± 0.84 (9)</td>
<td>0.015 ± 0.007 (9)</td>
</tr>
<tr>
<td>17.5 mg/l NO₃</td>
<td>1.35 ± 0.20 (5)</td>
<td>2.06 ± 0.46 (5)</td>
<td>0.021 ± 0.012 (5)</td>
</tr>
<tr>
<td>50 mg/l NO₃</td>
<td>1.87 ± 1.59 (8)</td>
<td>2.27 ± 0.95 (10)</td>
<td>0.020 ± 0.011 (8)</td>
</tr>
<tr>
<td>150 mg/l NO₃</td>
<td>1.63 ± 0.82 (9)</td>
<td>2.12 ± 1.09 (8)</td>
<td>0.015 ± 0.009 (9)</td>
</tr>
<tr>
<td>450 mg/l NO₃</td>
<td>1.20 ± 0.51 (6)</td>
<td>1.89 ± 0.80 (6)</td>
<td>0.017 ± 0.011 (6)</td>
</tr>
<tr>
<td>900 mg/l NO₃</td>
<td>1.94 ± 1.09 (7)</td>
<td>2.07 ± 1.05 (7)</td>
<td>0.012 ± 0.006 (7)</td>
</tr>
</tbody>
</table>

**Note.** Values represent mean ± SD; () = n.
shown indications that both nitrate and nitrite can traverse the placenta (Brunning-Fann and Kaneene, 1993; Gruener and Shuval, 1973; Manassaram et al., 2006), it seems that blood concentrations in the fetuses may be markedly lower than those in the dams (Gruener and Shuval, 1973). Another possible explanation for the lack of effects on steroidogenesis and histopathology in this study compared with the studies performed by Panesar and Chan (2000) and Pant and Srivastava (2002) is the much shorter dosing period in the current study and the difference in age of the animals at the time of dosing.

The lack of effects of in utero nitrate exposure on the selected endpoints indicate that nitrate does not induce anti-androgenic effects in male fetuses similarly to the anti-androgenic effect seen for certain phthalates (Borch et al., 2004, 2006).

Panesar (1999) and Panesar and Chan (2000) tested nitrate and nitrite in vitro in mouse Leydig tumor cells (MLTC-1) and found that both inorganic nitrate and inorganic inhibited steroidogenesis. Both nitrite and nitrate can endogenously be transformed to nitric oxide (NO) (Ellis et al., 1998) and Panesar (1999) and Panesar and Chan (2000) suggested that the inhibitory effects of nitrate and nitrite are through the action of the metabolite nitric oxide (NO), which is an inhibitor of steroid hormone synthesis (Cymeryng et al., 1998; Kostic et al., 1998; Masuda et al., 1997; Natarajan et al., 1997; Welch et al., 1995). In the current study neither nitrate nor nitrite had any dose-related effect on the production of testosterone or estradiol in the human adrenocortical carcinoma cell line (H295R) (Fig. 2). A significant decrease in testosterone production was seen for both compounds at 5\mu M. However, based on the lack of effect both at the other concentrations and also on the other endpoints in the current study, we cannot conclude anything from this data point. Furthermore, the in vitro results are in good agreement with the in vivo results in our study. The difference between the results from the previous experiments by Panesar (1999) and Panesar and Chan (2000), showing an inhibitory effect of inorganic nitrate on steroidogenesis, and the results from the present study, could be related to the different cell lines used. Furthermore, we do not know if the cell line used in the current study is able to metabolize nitrate and nitrite to NO.

Nitrate has previously been demonstrated by Zaki et al. (2004) to affect thyroid function in male rats exposed to potassium nitrate in the drinking water for 5 months. The effects of the nitrate exposure included reduction in total T3 levels at 150 and 500 mg/l and total T4 levels at 500 mg/l (Zaki et al., 2004). Esciocak et al. (2005) dosed adult female rats with sodium nitrate in drinking water for 30 weeks and found histological changes on the thyroid gland in animals exposed to nitrate at 250 and 500 mg/l. They also found reductions in total T3, free T4, and thyroid-stimulating hormone in serum at a nitrate dose of 50, 250, and 500 mg/l, but no significant effect on total T4 at the same doses. The only significant effect found on total T4 was an elevation of the total T4 level at 100 mg/l nitrate (Esciocak et al., 2005). In the present study, no effects of the nitrate treatment were observed on either plasma levels of total T4 or histopathology of the thyroid gland from the dams. This may be due to the much shorter exposure period used in this study compared with the studies done by Zaki et al. (2004) and Esciocak et al. (2005). In female fetuses T4 was significantly increased in the highest dose group. However the result is highly questionable as it was only possible to measure T4 above the detection limit in three out of six samples in the 900 mg/l group and furthermore, T4 was under the detection limit in all other groups. More studies are needed to evaluate whether the limited indication for increased T4 in female fetuses is true or a chance finding.

Nitrate and nitrite were also tested in the T-screen assay (Hohenwarter et al., 1996). No effects of either compound were observed. These results suggest that nitrate and nitrite do not exert their action through the thyroid receptor. A proposed mechanism in the literature is that nitrate and nitrite exerts their effect on the thyroid gland by inhibiting iodine uptake (Zaki et al., 2004), after they have been converted to nitric oxide. This kind of effect is not one that can be detected in the T-screen assay.

In conclusion, there were no consistent indications in the present study that nitrate induces anti-androgenic effects in male fetuses or that prenatal nitrate exposure affects the thyroid axis. The present study design differs from the animal studies performed by Panesar and Chan (2000) and Pant and Srivastava (2002) in which longer dose schemes were employed and the animals were dosed at a different age. Furthermore in those studies the animals were exposed directly whereas in our study the fetuses were indirectly exposed in utero. We cannot rule out the possibility that a longer dose period and/or direct exposure of pups in the time after birth can lead to decreased testosterone or affect the thyroid axis. Based on the above-mentioned studies, there is sufficient background for considering nitrate as a potential endocrine disruptor at dose levels close to the current.
regulatory limits in drinking water. Because the developmental period is an especially sensitive period, a more comprehensive study with long-term exposure before and during pre- and postnatal development would be relevant.

FUNDING

Danish Environmental Protection Agency (contract May 3, 2007).

ACKNOWLEDGMENTS

We would like to thank Louise Hass Madsen, Dorte Hansen, Birgitte Moller Plesning, Ulla El-Baroudy, Karen Roswall, Merete Lykkegaard, Vibeke J. Kjaer, Heidi Letting, and Morten Andreassen for their excellent technical assistance.

REFERENCES


