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ORIGINAL RESEARCH

Nitrate in Drinking Water and Time to Pregnancy or Medically Assisted Reproduction in Women and Men: A Nationwide Cohort Study in the Danish National Birth Cohort

Ninna Hinchely Ebdrup[1–3], Ulla Breth Knudsen[1,3], Jörg Schullehner[2,4], Linn Håkonsen Arendt[2,5], Zeyan Liew[6,7], Julie Lyngsø[2,5], Bjørn Bay[1,8], Pernille Jul Clemmensen[2], Torben Sigsgaard[2,9,10], Birgitte Hansen[4], Cecilia Høst Ramlau-Hansen[2]

[1]Department of Obstetrics and Gynecology, Horsens Regional Hospital, Horsens, Denmark; [2]Department of Public Health, Aarhus University, Aarhus, Denmark; [3]Department of Clinical Medicine, Aarhus University, Aarhus, Denmark; [4]Geological Survey of Denmark and Greenland, Aarhus, Denmark; [5]Department of Obstetrics and Gynecology, Aarhus University Hospital, Aarhus, Denmark; [6]Department of Environmental Health Sciences, Yale School of Public Health, New Haven, CT, USA; [7]Yale Center for Perinatal, Pediatric, and Environmental Epidemiology, Yale School of Public Health, New Haven, CT, USA; [8]Maigaard Fertility Clinic, Aarhus, Denmark; [9]Centre for Integrated Register-based Research at Aarhus University, Aarhus, Denmark; [10]Danish Big Data Centre for Environment and Health (BERTHA), Aarhus University, Aarhus, Denmark

Correspondence: Ninna Hinchely Ebdrup, Department of Obstetrics and Gynecology, Horsens Regional Hospital, Regionshospitalet Horsens, Sundvej 30, Horsens, 8700, Denmark, Tel +4528472111, Email ninebd@rm.dk

Purpose: No studies have investigated if drinking water nitrate affects human fecundity. Experimental studies point at detrimental effects on fetal development and on female and male reproduction. This cohort study aimed to explore if female and male preconception and long-term exposure to nitrate in drinking water was associated with fecundability measured as time to pregnancy (TTP) or use of medically assisted reproduction (MAR) treatment.

Methods: The study population consisted of pregnant women recruited in their first trimester in 1996–2002 to the Danish National Birth Cohort. Preconception drinking-water nitrate exposure was estimated for the pregnant women (89,109 pregnancies), and long-term drinking water nitrate exposure was estimated from adolescence to conception for the pregnant women (77,474 pregnancies) and their male partners (62,000 pregnancies) by linkage to the national drinking water quality-monitoring database Jupiter. Difference in risk of TTP >12 months or use of MAR treatment between five exposure categories and log-transformed continuous models of preconception and long-term nitrate in drinking water were estimated. Binominal regression models for risk ratios (RR) were adjusted for age, occupation, education, population density, and lifestyle factors.

Results: Nitrate in drinking water (median preconception exposure: 1.9 mg/L; median long-term exposure: 3.3 mg/L) was not associated with TTP >12 months or use of MAR treatment, neither in the categorical nor in the continuous models.

Conclusion: We found no association between preconception or long-term exposure to drinking water nitrate and fecundability.

Keywords: environmental pollutant, subfecundity, time to pregnancy, public health, groundwater

Introduction

Infertility, ie, the inability to conceive after 12 months of regular intercourse, affects one in six couples worldwide. Exposure to environmental pollutants may be a contributing factor apart from age, reproductive diseases in men and women and lifestyle factors.[1–3] A couple's fecundability is the biological ability to conceive and is often measured as the waiting time to pregnancy (TTP), eg, the number of menstrual cycles it takes to conceive.[4]

Nitrate is a widespread drinking water pollutant.[5,6] Although evidence from animal studies suggests an impairment of reproduction and fetal development following nitrate exposure,[7–13] limited human research has been undertaken regarding the potential impact on reproductive health. The evidence from experimental studies calls for more research in

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humans of low dose every day exposure to nitrate, as studies have mainly been conducted in rodents and at exposure levels far above the drinking water standard set by the World Health Organization.[14] Nitrate partly converts to nitrite, which can be endogenously transformed to N-nitroso compounds (NOCs) with DNA damaging potential. The formation of DNA damaging substances may have teratogenic and hormone disruptive effects in humans. Adverse birth outcomes were found at nitrate exposures below the drinking water standard, and thyroid and steroid imbalance have been speculated to potentially harm human reproductive function.[14–21] Further nitrate after reduction to nitrite can bind to hemoglobin and form methaemoglobin.[22] Methaemoglobin interferes with the oxygen-carrying capacity of the blood and might cause hypoxia in fetuses.[23] NOC formation and methaemoglobinemia are the suspected biological mechanisms potentially affecting reproduction in humans.[15,24–31] Humans are exposed to nitrate and NOCs throughout life and during susceptible reproductive windows. The current drinking water standard of nitrate is set at 50 mg/L,[16] a level above which there is a risk of acute infant methemoglobinemia. However, lower doses may also cause long-term health effects and the question remains whether the drinking water standard safeguards our reproductive health.[32,33]

To the best of our knowledge, no human studies exist on the potential effects of nitrate in drinking water on measures of fecundity.[17] We studied the association between exposure to preconception and long-term nitrate in drinking water and fecundability measured as waiting time to pregnancy (TTP) and use of medically assisted reproductive (MAR) treatment. We hypothesized that couples with higher nitrate exposure from drinking water had a higher risk of TTP >12 months and MAR treatment to conceive.

Materials and Methods

Study Population

This cohort study is based on all pregnant women participating in the Danish National Birth Cohort (DNBC) in 1996–2002 (n=91,380). We included 89,109 pregnancies and excluded pregnancies with missing exposure or covariate information according to the flow diagram (Figure 1). The DNBC had an estimated participation rate of 60% and included 30% of all pregnant women in Denmark during the study period.[34] Women were enrolled around gestational week 11 (SD 3.4) and provided information on lifestyle factors, health status, TTP, and potential use of MAR treatment. Computer-assisted telephone interviews were conducted in the first trimester, irrespective of whether the pregnancy ended in the birth of a child or pregnancy loss.[35] We linked information on age, residence, and vital status across national registries[36] using the unique personal identification number registered in the Danish Civil Registration System (CRS) and assigned to all Danish residents at birth or immigration.[37] The CRS contains current and historical information on sex, birthdate, vital status, spouse, and residence, including date of moving, immigration, and emigration. To study the potential effects of exposure to nitrate in drinking water in men, we linked the male partner to the female partner included in the DNBC by the unique identifier using registered information for married couples and cohabiting couples with mutual children. We also included the male partner in cohabiting couples with less than 15 years of age difference without mutual children in the CRS and not in close family relationship with each other. Using this algorithm, we were able to link 62,000 (70%) of the pregnancies included in the DNBC to a male partner. The CRS is recognized for its practically complete registration due to the general acceptance of registration requested by law, and data recorded in the CRS are considered to have high validity.[37]

Exposure

In Denmark, drinking water originates from groundwater, where nitrate concentrations vary mainly due to differences in agricultural nitrogen fertilization and management as well as the geology and geochemistry characteristics of the subsurface.[38,39] Household-level data have been used to estimate individual-level nitrate exposure from drinking water across several studies of nitrate associated with adverse health outcomes.[15,33,40] In brief, to determine nitrate concentrations in drinking water at each household, we linked the national drinking water quality monitoring database[41] to the geocoded residential history for every person registered in the CRS from 1978 onwards[37] by using digitized water supply areas.

We excluded non-detects exceeding the upper level of detection (1 mg/L) and calculated annual nitrate levels for each household. For years without nitrate measurements, we imputed concentrations by interpolation if there was

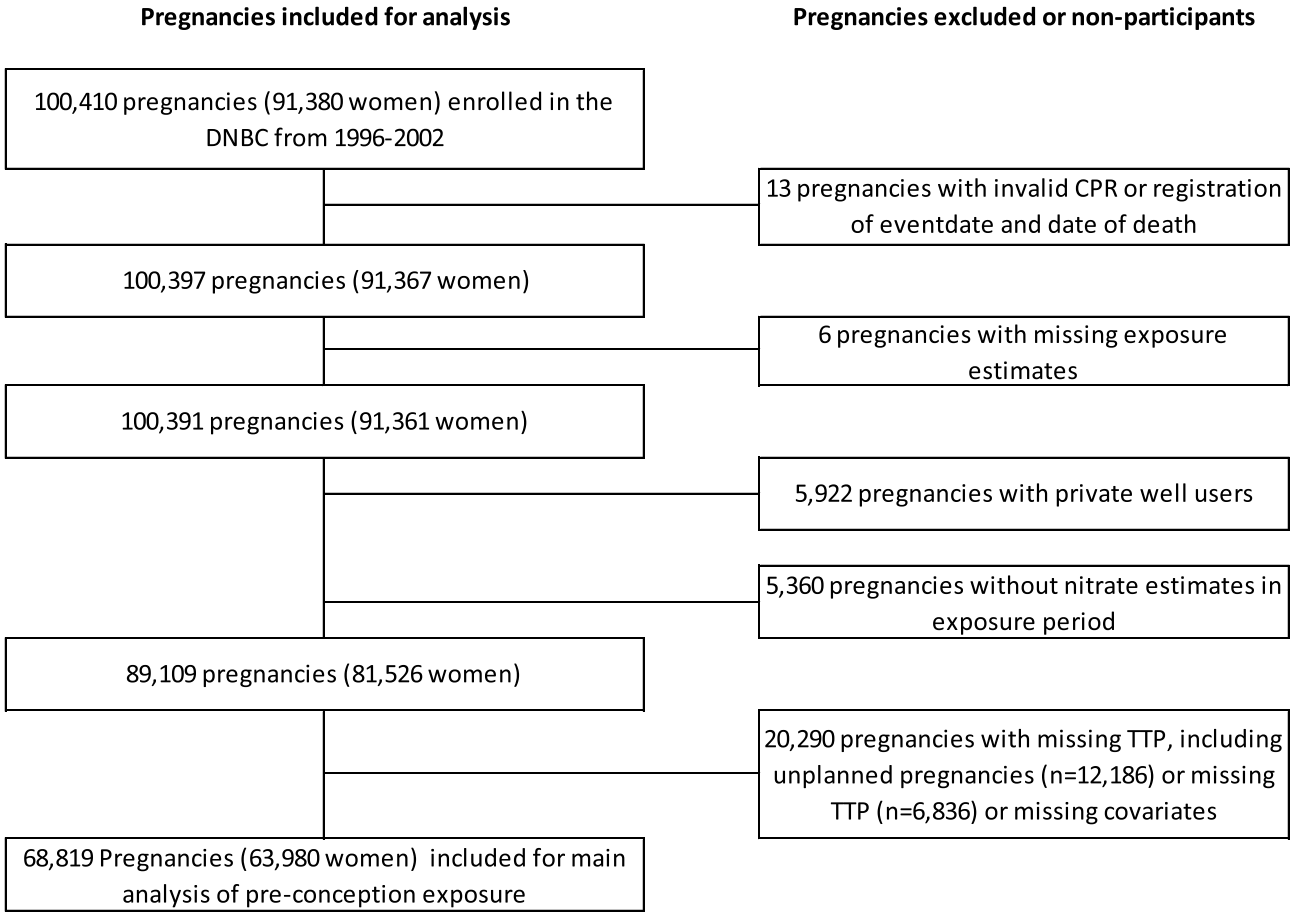

Figure 1 Flow chart of the study population from the Danish National Birth Cohort (1996–2002).
Abbreviations: DNBC, Danish National Birth Cohort; CPR, personal identification number; TTP, time to pregnancy.

a measurement available within 3 years.[42] We excluded private well users because they are monitored far less than public waterworks in Denmark.[43] We calculated time-weighted average exposures while taking into account both temporal changes of nitrate levels at the households and changes of residency. For a more detailed description of the approach, see Schullehner et al.[38,42,43] We calculated each woman's average nitrate exposure from 2 years prior to conception (hereinafter "preconception exposure"). For 83% of the DNBC couples, the couples shared the preconception exposure estimates because the exposure levels were estimated based on the residential history of the woman. To study separately the potential effects in women and men and to cover the period from adolescence until conception and gain larger exposure contrast, we calculated long-term individual nitrate estimates for both sexes (n=74,774 women, n=62,000 men). The long-term exposure window was defined from January 1, 1978 (or from the date of birth if the women or men were born after January 1, 1978) until date of conception (hereinafter "female and male long-term exposure").

For the preconception exposure assessment, we included exclusively participants with complete exposure data. For the long-term exposure assessments, we allowed participants to have missing exposure information for a maximum of 50% of the exposure window.[33]

Outcome

In the first DNBC interview, which was conducted around gestational week 15, women reported their TTP into five predefined categories (less than 1 month, after 1–2 months, after 3–5 months, after 6–12 months, more than 12 months). MAR treatment was included in the questionnaire if the women reported a TTP of 6 months or longer. In the present study, we defined the outcome as TTP >12 months or use of MAR treatment (reference TTP ≤12 months). Women also

reported if their pregnancy was planned. In the main analysis, we excluded unplanned pregnancies (14%) as they were considered to include pregnancies of highly fecund or subfecund couples.[44]

Covariates

We made an *a priori* decision on which covariates to include by reviewing the literature and using directed acyclic graphs (Supplemental Figure 1).[45] We included age, body mass index (BMI), smoking, alcohol consumption, occupation, education, and population density (as a proxy for area and environment)[46] as listed in Table 1. We modeled age and population density as restricted cubic splines. Level of education and occupation at conception were derived from Statistics Denmark.[47,48] Age and lifestyle variables were available from the DNBC interviews. Lifestyle was included to improve the precision as we expected BMI, smoking, and alcohol to be strongly associated with the outcome.

Table 1 Characteristics of the Female Study Population from the Danish National Birth Cohort (1996–2002) According to Preconception Drinking Water Nitrate

	Nitrate Concentration (mg/L)				
	≤1	>1 - ≤2	>2 - ≤5	>5 - ≤25	>25
Characteristics					
Total study population[a], pregnancies, N (%)	24,050 (27.0)	26,162 (29.4)	25,375 (28.5)	10,720 (12.0)	2802 (3.1)
Age at conception in years, mean (SD)	29.71 (4.28)	29.99 (4.24)	29.76 (4.39)	29.42 (4.45)	29.70 (4.39)
Age at conception, n (%)					
<20 years	235 (1.0)	194 (0.7)	275 (1.1)	140 (1.3)	39 (1.4)
≥20 to <25 years	2842 (11.8)	2642 (10.1)	3070 (12.1)	1543 (14.4)	348 (12.4)
≥25 to <30 years	10,095 (42.0)	10,820 (41.4)	10,333 (40.7)	4373 (40.8)	1117 (39.9)
≥30 to <35 years	8139 (33.8)	9256 (35.4)	8586 (33.8)	3469 (32.4)	959 (34.2)
≥35 to ≤40 years	2489 (10.3)	2926 (11.2)	2805 (11.1)	1080 (10.1)	311 (11.1)
>40 years	250 (1.0)	324 (1.2)	306 (1.2)	115 (1.1)	28 (1.0)
Highest education, n (%)					
Primary school	2043 (8.5)	1668 (6.4)	1952 (7.7)	982 (9.2)	271 (9.7)
High school or vocational	10,035 (41.7)	8784 (33.6)	9435 (37.2)	4803 (44.8)	1236 (44.1)
Basic education	1975 (8.2)	1772 (6.8)	1826 (7.2)	766 (7.1)	210 (7.5)
Middle education	7689 (32.0)	8711 (33.3)	8202 (32.3)	3272 (30.5)	866 (30.9)
Higher education or PhD	2278 (9.5)	5185 (19.8)	3920 (15.4)	879 (8.2)	≥209 (7.5)
Missing	30 (0.1)	42 (0.2)	40 (0.2)	18 (0.2)	≤5 (.)
Occupation, n (%)					
Unemployed[b]	2417 (10.0)	2310 (8.8)	2476 (9.8)	1235 (11.5)	322 (11.5)
Student	1833 (7.6)	3037 (11.6)	2422 (9.5)	772 (7.2)	194 (6.9)
Employee unspecified income	1765 (7.3)	1717 (6.6)	1685 (6.6)	810 (7.6)	226 (8.1)
Employee with low income	8906 (37.0)	7471 (28.6)	8316 (32.8)	4104 (38.3)	1055 (37.7)
Employee with middle income	5674 (23.6)	6327 (24.2)	5875 (23.2)	2300 (21.5)	576 (20.6)
Chief executive or employee with high income	2627 (10.9)	4432 (16.9)	3772 (14.9)	1107 (10.3)	318 (11.3)
Owner of business	431 (1.8)	513 (2.0)	448 (1.8)	222 (2.1)	49 (1.7)
Other	397 (1.7)	355 (1.4)	381 (1.5)	170 (1.6)	62 (2.2)
Population density[c] <250 meters, mean number of people (SD)	485 (592)	1154 (1346)	1094 (1289)	577 (856)	401 (465)
Smoking in pregnancy, cigarettes/day, n (%)					
Non-smoker	16,658 (69.3)	18,179 (69.5)	17,080 (67.3)	7102 (66.3)	1949 (69.6)
Previous smoker in early pregnancy	1819 (7.6)	2416 (9.2)	2370 (9.3)	900 (8.4)	185 (6.6)
1–9	2071 (8.6)	2040 (7.8)	2184 (8.6)	1000 (9.3)	230 (8.2)
10–14	990 (4.1)	976 (3.7)	1158 (4.6)	554 (5.2)	163 (5.8)
≥15	601 (2.5)	611 (2.3)	729 (2.9)	402 (3.8)	91 (3.2)
Missing	1911 (7.9)	1940 (7.4)	1854 (7.3)	762 (7.1)	184 (6.6)

(*Continued*)

Table 1 (Continued).

	Nitrate Concentration (mg/L)				
	≤1	>1 - ≤2	>2 - ≤5	>5 - ≤25	>25
Weekly alcohol consumption, units/week, n (%)					
0	12,100 (50.3)	12,972 (49.6)	13,265 (52.3)	5745 (53.6)	1483 (52.9)
0.5–3.5	9554 (39.7)	10,660 (40.7)	9729 (38.3)	3986 (37.2)	1081 (38.6)
4–7	432 (1.8)	532 (2.0)	460 (1.8)	195 (1.8)	≥42 (1.5)
≥7.5	37 (0.2)	46 (0.2)	54 (0.2)	18 (0.2)	≤ 5 (.)
Missing	1927 (8.0)	1952 (7.5)	1867 (7.4)	776 (7.2)	187 (6.7)
Body mass index, kg/m^2, n (%)					
Underweight	878 (3.7)	1115 (4.3)	1150 (4.5)	436 (4.1)	113 (4.0)
Normal weight	14,407 (59.9)	16,938 (64.7)	15,957 (62.9)	6176 (57.6)	1629 (58.1)
Overweight	4511 (18.8)	4179 (16.0)	4268 (16.8)	2158 (20.1)	547 (19.5)
Obesity	2033 (8.5)	1601 (6.1)	1746 (6.9)	1023 (9.5)	292 (10.4)
Missing	2221 (9.2)	2329 (8.9)	2254 (8.9)	927 (8.6)	221 (7.9)
Gravidity[d], total pregnancies, n (%)					
Nulligravidae	9 (0.0)	12 (0.0)	17 (0.1)	11 (0.1)	≤ 5 (.)
Primigravidae	8163 (33.9)	9845 (37.6)	9330 (36.8)	3626 (33.8)	≥885 (31.5)
Multigravidae	≥15,872 (66.0)	16,305 (62.3)	≥16,021 (63.1)	≥7077 (66.0)	1911 (68.2)
Missing	≤ 5 (.)	0 (0.0)	≤ 5 (.)	≤ 5 (.)	0 (0.0)
Parity[d], n (%)					
Nullipara	10,736 (44.6)	13,686 (52.3)	13,102 (51.6)	4913 (45.8)	1178 (42.0)
Multipara	≥13,308 (55.3)	12,476 (47.7)	≥12,266 (48.3)	≥5801 (54.1)	1624 (58.0)
Missing	≤ 5 (.)	0 (0.0)	≤ 5 (.)	≤ 5 (.)	0 (0.0)
Previous spontaneous pregnancy loss[d], n (%)					
No prior loss	19,215 (79.9)	20,980 (80.2)	20,504 (80.8)	8564 (79.9)	2205 (78.7)
1–2	4670 (19.4)	5028 (19.2)	4704 (18.5)	2075 (19.4)	573 (20.4)
≥3	≥159 (0.7)	154 (0.6)	≥160 (0.6)	≥75 (0.7)	24 (0.9)
Missing	≤5 (.)	0 (0.0)	≤ 5 (.)	≤ 5 (.)	0 (0.0)

Notes: According to data protection regulations, values below 5 may not be reported (GDPR, Regulation (EU), 2016/679 of 25 May 2018). In case of values below 5, pseudo numbers were estimated as the value nearest to the actual count > or <5. [a]N=89,109. [b]Unemployed: Social security benefits, disability pension, or state education grant. [c]Population density: number of people living 250 meters or less from the woman's residence at the time of pregnancy, based on distance between geocoded address points from the Residence Database.[46] Rounded to whole numbers. [d]Reproductive history on all included pregnancies.[77]
Abbreviation: SD, standard deviation.

Statistical Analyses

To address our aim, we used two different analytical models. In the first analytical model for the analyses of preconception and long-term nitrate exposures, we applied binominal regression models and estimated adjusted risk ratios (RR) with 95% confidence intervals (CIs) comparing the risk of TTP >12 months or MAR treatment between five categories of drinking water nitrate exposure (mg/L NO_3^-). We categorized drinking water nitrate into five *a priori* defined groups (≤ 1 mg/L (reference), >1 - ≤2 mg/L, >2 - ≤5 mg/L, >5 - ≤25 mg/L, and >25 mg/L).[15] The continuous variable was log-transformed and modeled using restricted cubic splines with four knots.[49]

We conducted several sensitivity analyses. In the first sensitivity analysis, we studied MAR treatment as the outcome (reference: no MAR treatment). In the second and third sensitivity analyses, we introduced unplanned pregnancies to the group with the longest TTP (TTP >12 months or MAR treatment) and the group with the shortest TTP (the reference), respectively. The couple's lifestyle may be reflected in the individual's lifestyle. However, as female lifestyle is only a proxy of male lifestyle, we omitted female lifestyle in a fourth sensitivity analysis of male long-term nitrate exposure. To reduce the risk of exposure misclassification in the long-term exposure analyses, the study population was restricted in the fifth and sixth sensitivity analyses to those who had lived 75% of the exposure window at residences with a nitrate estimate.

In the second analytical model, we estimated the mean differences in TTP (days) between the five categories of nitrate exposure compared to the reference concentration below 1 mg/L using linear regression with robust variance to account for

departures from normality. We used simple imputation relying on clinical knowledge[50,51] to impute TTP based on the categories given from the DNBC interview (TTP <1 month = TTP 0 month, TTP 1–2 months = TTP 1.5 months, TTP 3–5 months = TTP 4 months, TTP 6–12 months = TTP 9 months, TTP above 12 months = TTP 13 months, MAR treatment = TTP 18 months).

In quantitative bias analyses, we addressed the risk of bias due to selection on fetal survival in the inclusion to the DNBC.

All models were fitted using robust variance estimation to account for dependencies between pregnancies by the same individual. There was no statistically significant interaction between any of the exposures and adjusting variables. Continuous variables (age and population density) were included as restricted cubic splines with four knots.

We conducted the statistical analyses and modeling of the exposure by use of R (version 3.6; R Development Core Team) and Stata, version 15.0 (StataCorpLP, College Station, TX, USA).

Ethical Considerations

The participating women in the DNBC gave written consent. The data were pseudo-anonymized, and Aarhus University approved the study as Data Controller (No. 2015-57-002, project No. 1643). No ethical approval from the Danish Ethics Committee was required for this study in accordance with Danish legislation.

Results

Of the 100,410 pregnancies in the DNBC, we identified 89,109 as the female study population with the characteristics presented in Table 1. We included 68,819 (69%) pregnancies with sufficient exposure data and TTP data for the main analysis of preconception exposure (Figure 1). Among these, 11,626 (17%) women had a TTP >12 months or needed MAR treatment to conceive. The median preconception nitrate exposure was 1.9 mg/L (95% prediction interval (PI): 0.2–16.1 mg/L).

In the analyses of long-term nitrate exposure, the right skewed exposure distribution was 3.3 mg/L (95% PI: 0.5–23.8) (median among women) and 3.3 mg/L (95% PI: 0.4–24.5) (median among men). The mean time of long-term exposure was 22 years (16–25 years) for women and 21 years (18–25 years) for men, corresponding to follow-up from age 8 in women and age 11 in men. The characteristics of the male study population are presented in Supplemental Table 1.

Table 2 shows adjusted RRs for TTP >12 months or MAR treatment according to preconception exposure and female and male long-term exposures. Overall, we found no higher risk of TTP >12 months or MAR treatment with increasing levels of nitrate. In the analysis of preconception exposure, drinking water nitrate was associated with a slightly lower risk of TTP >12 months or MAR treatment at nitrate levels 1–5 mg/L compared to the reference category (below highest level of detection, 1 mg/L). Nitrate in drinking water was not associated with TTP >12 months or MAR treatment in the long-term analyses.

When using cubic splines to assess the association (Figure 2), we found no differences in the adjusted RRs according to levels of drinking water nitrate, neither in the preconception nor in the long-term exposure models.

The estimated mean differences in TTP ranged from −15 days (96% CI: −18, −12) to −2 days (95% CI: −9, 6) when we compared the four preconception exposure categories above 1 mg/L NO_3^- to the TTP of the reference group (mean TTP 164 days (95% CI: 162, 167)) (Supplemental Table 2).

The sensitivity analyses of both preconception exposure and long-term exposure supported the findings of the main analyses (Supplemental Tables 3–8). Bias analyses were reported in Supplemental Tables 9–13.

Discussion

Key Findings

The findings did not support our hypothesis of higher risk of TTP >12 months or MAR treatment with increasing levels of drinking water nitrate, neither for the preconception exposure nor for the long-term exposure in women or men. When we modelled the exposure as a continuous variable, no difference in risk was found. In the categorical analyses, we found slightly lower risk estimates in the lowest categories of exposure in the analysis of preconception nitrate in drinking

Table 2 Adjusted Risk Ratios for the Association Between Nitrate in Drinking Water and Time to Pregnancy Above 12 Months or Use of Medically Assisted Reproduction (MAR) Treatment, The Danish National Birth Cohort (1996–2002)

	Female Preconception Exposure			Female Long-Term Exposure			Male Long-Term Exposure		
	n = 68,819 Pregnancies			n = 60,065 Pregnancies			n = 49,202 Pregnancies		
NO_3^- (mg/L)	Pregnancies n (%)	TTP >12 Months/ MAR, n (%)[a]	aRR (95% CI)[b]	Pregnancies, n (%)	TTP >12 Months/ MAR, n (%)[a]	aRR (95% CI)[b]	Pregnancies, n(%)	TTP >12 Months/ MAR, n (%)[a]	aRR (95% CI)[c]
≤1	18,622 (27.1)	3450(18.5)	Ref (1)	5956(9,9)	1010 (17.0)	Ref (1)	4976 (10.1)	867 (17.4)	Ref (1)
>1 to ≤2	20,272 (29.5)	3230 (15.9)	0.93 (0.89, 0.98)	12,793 (21.3)	2061 (16.1)	0.94 (0.88, 1.01)	10,358 (21.1)	1789 (17.3)	0.99 (0.93, 1.07)
>2 to ≤5	19,496 (28.3)	3091 (15.9)	0.91 (0.87, 0.95)	23,139 (38.5)	3920 (16.9)	0.96 (0.90, 1.03)	18,759 (38.1)	3404 (18.1)	1.03 (0.96, 1.10)
>5 to ≤25	8229 (12.0)	1443 (17.5)	0.96 (0.91, 1.01)	16,304 (27.1)	2660 (16.3)	0.93 (0.88, 1.00)	13,516 (27.5)	2322 (17.2)	0.97 (0.91, 1.04)
>25	2200 (3.2)	412 (18.7)	0.99 (0.91, 1.09)	1873 (3.1)	315 (16.8)	0.97 (0.87, 1.09)	1593 (3.2)	278 (17.5)	0.98 (0.87, 1.11)

Notes: Model was fitted using robust variance estimation (VCE) to account for dependencies of pregnancies by the same woman. [a]Time to pregnancy above 12 months or use of medically assisted reproduction (MAR) treatment, n (% proportion of total). [b]Adjusted for maternal age, education, occupation, population density, and maternal lifestyle (BMI, smoking, and alcohol) at time of conception. [c]Adjusted for paternal age and education at time of conception. Population density of maternal residence and maternal lifestyle factors (BMI, alcohol, and smoking) at time of conception.

Abbreviations: NO_3^-, nitrate concentration in drinking water; CI, confidence interval; Ref, reference.

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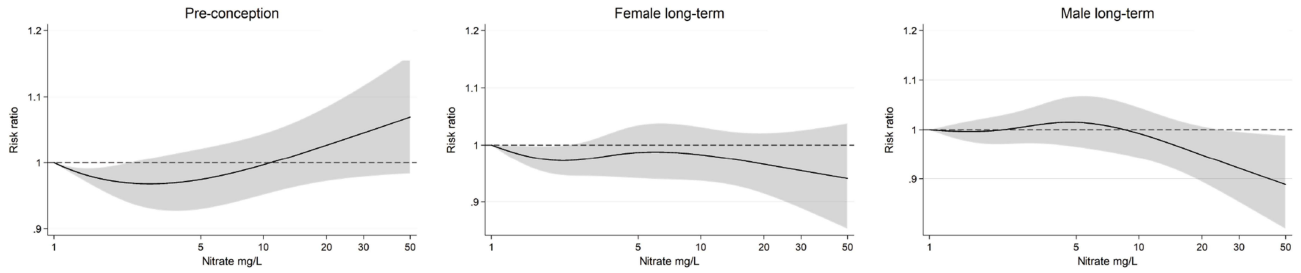

Figure 2 Adjusted risk ratios of time to pregnancy above 12 months or use of MAR treatment by drinking water nitrate exposure (log scale) with 1 mg/L as reference. Exposure below the highest detection limit 1 mg/L and above 50 mg/L is not shown but is included in the model. The gray scale area represents the confidence interval (CI). Female splines were adjusted for female age, education, occupation, population density, body mass index, smoking, and alcohol. Male splines were adjusted for male age and education and female population density, body mass index, smoking, and alcohol. Robust variance estimation (VCE) accounted for dependencies of pregnancies.

water and no difference in risk in the long-term models. In absolute measures, the mean difference in TTP in days was found to be clinically negligible.

Current Evidence

To our knowledge, no published studies exist on the association between drinking water nitrate and female or male infertility or fertility treatment, and the biological mechanism by which nitrate exposure may impair reproductive function remains unclear.[17] Nitrate may, after reduction to nitrite, form water-soluble teratogenic and endocrine disruptive compounds that may expose reproductive organs and developing fetuses.[52–54] In humans, nitrate exposures below 50 mg/L have previously been associated with thyroid dysfunction, neonatal death, fetal growth restriction, prematurity, and congenital malformations.[15,24–31] However, this suggestion of reproductive toxicity was not reflected in a case–control study from 1989 of fetal death of 286 women who had a pregnancy loss and 1391 controls with live births admitted to the same hospital. The exposure was estimated based on public water supply samples taken after the time of pregnancy loss and were below the drinking water standard. In a Danish cohort study of 652,810 women with the first recorded singleton pregnancy, the risk of stillbirth was not associated with nitrate below the drinking water standard.[24,40] The hypothesis of adverse methaemoglobin reproductive effects was suggested after evaluation of 25 women with pregnancy loss in the first trimester and higher levels of methaemoglobin.[23] Methaemoglobin associated pregnancy loss was not found in women with pregnancy loss compared to women with term delivery, by long-term measurements of serum methemeglobin levels in pregnancy.[55]

In animals, nitrite has been associated with impaired reproduction in mice, rats, guinea pigs, and cattle, with longer days to litter and perinatal death.[7,11,52,56] In utero exposure to nitrite has also shown trans-placental effects of mutagenic cell damage in hamster embryos.[53] Likewise, impaired male reproductive function has been reported at exposures to 31.5–122 mg/L NO_3^- in rats and to 214–427 mg/L NO_3^- in rabbits.[12,57] Nitrate has also been suspected of steroid hormone disruptive effects at nitrate exposures of 30.5 mg/L NO_3^-, although with inconsistent findings in experimental studies.[18,20,58] In addition other experimental studies in rodents found no reproductive harmful effects at toxic doses to nitrite and nitrate.[17]

Strengths and Limitations

A major strength of our study was the inclusion of a large study population with individual-level exposure assessments of both women and men. In a life course perspective, the peri-conceptual period, adolescence, and young adulthood are all critical biological periods. Exposure in these susceptible windows may have a lifelong and irreversible effect and may influence future reproductive health.[59] The female ovaries are fully developed in perinatal life and are thus exposed lifelong. In men, the testes remain dormant after organogenesis until puberty, in which spermatogenesis kick-starts the recurrent gamete production.[60]

The analyses of long-term nitrate exposure (ie, 21 years of exposure) covering from pre-puberty until conception (age 8–29 in women, age 11–32 in men) allowed us to investigate low-dose effects on later reproductive health, not previously tested in human

or animal models. We relied on a unique resource of longitudinally collected drinking water samples from certified laboratories.[38,39,41] Nitrate levels in drinking water in Denmark are without seasonal variation, and the bioavailability is considered steady, with continuous drinking water intake.[38,61] As a result, exposure changes are more likely due to change of address, which we accounted for by day-specific residential data. Further, we included confounding variables from interviews and national registries of high validity and completeness.[34,38,62] Thus, this nationwide study is a significant contribution to an overlooked field, despite the global exposure to nitrate and the potential influence on public health as a modifiable risk factor.

The study included only women who obtained a pregnancy lasting at least to the end of first trimester. Around 30% of all pregnancies are lost in the first months of pregnancy, with the highest percentage in the earliest weeks of fetal life.[63,64] The DNBC included women when they consulted their general practitioner, with a mean gestational age at enrollment of 11 completed weeks. Even though we also included women experiencing pregnancy loss after inclusion, we could not include the earliest of pregnancy losses. Moreover, the inclusion of pregnancies conceived after MAR treatment was not representative, as 30% of couples receiving MAR treatment do not get a child.[65] In this regard, the DNBC comprises a cohort of couples with proven fertility and fetal survivors. This may have led to biased results, as collider stratification bias may have been present if both high nitrate exposure and inability to conceive are associated with selection into the study. Collider stratification bias due to selection has long been an issue of concern in studies of environmental exposures, and even inverse associations have been speculated to be due to selection of survivors.[66,67] To quantify the magnitude of potential selection bias towards the null we conducted bias analyses for the preconception exposure as presented in the Supplemental Material Tables 12 and 13. We found that in scenarios with selection >30% and high degree of effect modification by individual factors related to endogenous nitrosation capacity, biased results might have masked reproductive harmful effects.

We relied on exposure assessments at residential level and estimated nitrate in drinking water as a proxy for endogenous nitrate exposure, as biological samples were unavailable. We expect only moderate degrees of non-differential misclassification of exposure, which is likely to have produced bias towards the null. In a Swedish study, 70% of cold tap water was consumed at home,[68] and the Danish population consumes the least bottled water in Europe.[69,70] In addition, we expect that most people have jobs and homes in geographical proximity, and their drinking water source will thereby originate from the same area.

Fecundability is a couple concept, and the preconception exposure assessment based on female residence may be identical to the male preconception exposure. The two-year exposure window was set after discussions in the author group of a clinically relevant preconception exposure window, while considering the rather constant nitrate level over the years. In our study, the couple's preconception exposures were highly correlated, as 83% of the couples shared residence at the time of conception. Further, one would expect the couples to share lifestyle and other potential confounding factors.[71,72] The risk of misclassification cannot be ruled out, and it is however reassuring that the sensitivity analysis solely including male covariates in the model (Supplemental Table 6) did not change the estimates significantly.

We relied on retrospectively reported TTP, although with short recall time as it was reported in early pregnancy. In Denmark, couples are offered MAR treatment free of charge for up to three in vitro attempts or a child. In a validation study, the self-reported MAR treatment in the DNBC was compared to that in the Danish national registries, and a positive predictive value of 88% was reported.[73] Thus, we expect the risk of misclassification to be of little importance.[74] Further, we do not anticipate a potential misclassification to be differential because participants are expected to be unaware of their nitrate exposure. We had no information on other aspects of family planning, such as timing and frequency of intercourse or contraceptive planning, but we do not expect these to be associated with nitrate levels.

The women with unplanned pregnancies could not report their TTP and were excluded from the main analyses. They may represent either the most fecund or the least fecund women. However, in the sensitivity analyses, in which we included women with unplanned pregnancies in first the TTP >12 months category and second the reference category, we found no changes from the main analysis.

Even though we were able to adjust for important potential confounders and precision variables, including socioeconomic position, lifestyle factors, and environmental factors, we cannot rule out residual confounding, eg, by nitrate in diet or other agricultural pollutants in drinking water. To address potential co-contamination of nitrate and other anthropogenic pollutants, ie, pesticides, we adjusted for population density as proxy for other environmental pollutants. Still, residual confounding of

other pollutants cannot be ruled out.[75] Nitrate is also found in green vegetables and cured meat, and diet may contain inhibitory antioxidants and vitamins that may protect against nitrate-associated adverse health effects.[76]

The generalizability of these results is subject to certain limitations, as we relied on a selected cohort of women and their male partners, who all obtained a pregnancy. Sterile and subfecund couples are underrepresented, as we condition on pregnancy, and the findings cannot be generalized to sterile people or exposure above the drinking water standard. We need novel strategies for recruiting couples to measure TTP in the general population when studying potential adverse effects of environmental exposures. Longitudinal measurements of TTP in populations of pregnancy planners and enrollment of pregnancies by the same mother will enable us to better understand potential sensitive windows and effects in susceptible populations.

Conclusion

Preconception and long-term drinking water nitrate exposure at comparatively low exposure levels were not associated with higher risk of TTP >12 months or MAR treatment in couples who obtained a pregnancy. Selection and exposure misclassification may have biased our results towards the null, and our findings need replication in the general population.

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Disclosure

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