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Daily sperm production: Application in studies of prenatal exposure to nanoparticles in mice

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ABSTRACT

We investigated the influence of maternal airway exposure to nanoparticulate titanium dioxide (TiO_2, UV-Titan) and carbon black (CB, Printex90), on male reproductive function in the two following generations. Time-mated C57BL/6J mice were exposed by inhalation to UV-Titan, or by intratracheal instillation with Printex90. Body and testicle weight, sperm content per g testicular parenchyma and daily sperm production (DSP) were assessed. The protocol for assessment of DSP was optimized for application in mice (C57BL/6J) and the influence of different parameters was studied. Maternal particulate exposure did not affect DSP statistically significantly in the F1 generation, although TiO_2 tended to reduce sperm counts. Overall, time-to-first F2 litter increased with decreasing sperm production. There was no effect on sperm production in the F2 generation originating after TiO_2 exposure. F2 offspring, whose fathers were prenatally exposed to Printex90, showed lowered sperm production. Furthermore, we report statistically significant differences in sperm production between mouse strains.

1. Introduction

Rapid advancement of nanotechnology emphasizes the need to investigate health effects of exposure to engineered nanoparticles and nanomaterials. In mice, engineered nanoparticles have been shown to adversely affect male reproductive function after exposure in adulthood [1,2] as well as after maternal exposure during foetal development [3,4]. Specifically, maternal subcutaneous injection of nano-sized titanium dioxide (TiO_2) particles during gestation resulted not only in TiO_2 particles aggregating in offspring testicular tissue after birth, but also in abnormal testicular morphology and lower daily sperm production in mice [3]. Also in mice, offspring seminiferous tissue and daily sperm production were adversely affected after maternal gestational exposure to nano-sized carbon black (CB) particles by intratracheal instillation [4]. That male reproductive function is sensitive to maternal gestational exposure to particles is supported by studies of particles generated from diesel engines. Daily sperm production (DSP) decreased in male rodents whose mothers inhaled diesel exhaust particles (DEP) or whole diesel exhaust during pregnancy [5–7]. Furthermore, maternal exposure to DEP increased the number of germline mutations in male offspring, and these mutations were transferred to the next generation in mice [8].

Observations of adverse effects on male reproductive function following prenatal exposure to particles with few associated compounds, as described above for TiO_2 and CB, indicate that prenatal exposure to particulates per se poses a threat to male reproductive function [3,4]. Elucidation of the relationship between maternal exposure to engineered nanoparticles and reproductive function of the male offspring is therefore important.

Semen samples cannot easily be obtained from rodents. Assessment of DSP is therefore a suitable method for evaluation of spermatogenesis in these species [9–11]. DSP is assessed by

Abbreviations: ANOVA, analysis of variance; CB, carbon black; DEP, diesel exhaust particles; DSP, daily sperm production; ESTR, expanded simple tandem repeats; HT, half testicle; SC/g, sperm content per g testicular parenchyma; SEM, standard error of the mean; WT, whole testicle.

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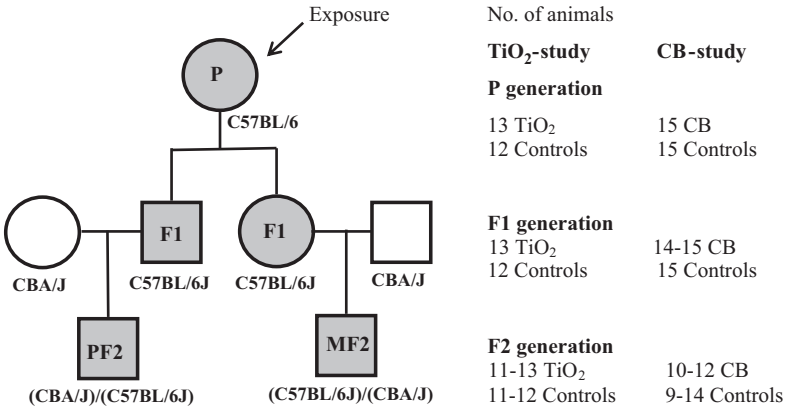

Fig. 1. Pedigree overview of the studies. Circles represent females and squares males. Grey symbols represent exposed pregnant mice and their descendants. White symbols represents unexposed CBA/J mice. In the parental (P) generation only pregnant females were used (C57BL/6J). For the F1 generation C57BL/6J females and males were cross mated with unexposed mature CBA/J mice. The F2 generation represents the male offspring of these crosses (MF2 – mothers were exposed in utero (C57BL/6J)/(CBA/J), PF2 – fathers were exposed in utero (CBA/J)/(C57BL/6J)).

homogenization of testicular tissue, followed by counting the number of spermatids surviving homogenization. The number of counted spermatids is then divided by a time factor expressing the duration of this homogenization resistant stage. Finally, the number is recalculated taking absolute testicle weight into account. The method was first used in the 1960s for calculation of DSP/g testicle parenchyma in bulls [12]. The method has since been applied in several other species, e.g. humans, boars, rabbits, mouse, and rat [9]. The mouse is a much used experimental animal within nanotoxicology. DSP has been the method of choice in assessments of male reproductive toxicity not only in adult but also developmental toxicity studies of nanosized particles [1–5,13].

Airway exposure to nanosized particles can occur during exposure to air pollution and at the work place. The effects of maternal exposure to engineered nanoparticles on sperm production in the male offspring have only been little studied, and to the best of our knowledge effects in the second generation remain to be investigated. Furthermore, even if mice are much used within reproductive nanotoxicology, application and optimization of the method in mice has to our knowledge never been thoroughly described in the scientific literature. The present study therefore aimed to: (1) describe and optimize the method of DSP assessment in mice and (2) examine the effects of maternal airway exposure during gestation to two types of engineered nanoparticles on DSP in the F1 and the F2 generations.

2. Materials and methods

2.1. Daily sperm production for use in mice

The method of DSP assesses the number of spermatids produced in the testicles per day and therefore provides one measure of the male reproductive function [9,14]. In short, the left testicle was dissected during necropsy and decapsulated, and the parenchyma was homogenized by IKA ULTRA TURRAX® T25 (disperser S25N-10G) in 0.05% triton X-100 homogenization buffer (0.9% NaCl and 0.05% triton X-100; Sigma®, Germany). Triton X-100 was added to reduce the amount of tissue debris, which might interfere during counting in haemocytometer [15]. Homogenization resistant (elongated) spermatids (stage 14–16) in the homogenate were stained by 0.04% Trypan blue (GURR®; Hopkin and Williams, England) in 1× phosphate buffer and after 30 min counted in haemocytometer (Bürker chamber, 0.0025 mm^2, depth 0.100 mm) under light microscope at 400× magnification. Samples were counted in two replicates, and each replicate was evaluated by averaging counts from 3 different squares, each with 16 fields. Counts from the two replicates were averaged and served as basis for calculation of the number of homogenization resistant spermatids per g testicle tissue (sperm content per g testicular parenchyma, SC/g). The total number of spermatids in the left testicle was then calculated by multiplication with the weight of the left testicle. DSP was then calculated by dividing the total number of elongated spermatids in the testicle with the time divisor value of 4.84. This number corresponds to the time (in days) which developing spermatids spend in stage 14–16 during spermatogenesis in the mouse [16].

To optimize the method as previously described for use in rats [9–11], several methodological steps were assessed in male C57BL/6J mice (Taconic Europe, Ejby, Denmark). This mouse strain has extensively used in particle- and nanotoxicology and therefore allows for interstudy comparison. Unless otherwise specified, the animals were killed by cervical dislocation 3 days after arrival from the breeder, testicles were excised and flash frozen immediately in liquid nitrogen and stored at −80 °C until analysis. The following steps were assessed.

2.1.1. Volume of homogenization buffer

Half (HT; ∼50 mg tissue) or whole (WT; ∼90 mg tissue) testicles were homogenized in 3 different volumes of homogenization buffer (HT: 2, 4 and 8 ml, WT: 6, 8 and 10 ml; animal age 70 and 210 days, respectively, $n = 5$).

2.1.2. Speed of the disperser and duration of homogenization

The speed of the disperser was varied from 11,000 to 24,000 rpm in 6 steps. For each speed, one HT in 4 ml of homogenization buffer was used. After 60 s of homogenization, 200 μl were aliquotted and homogenization continued, for a total of 90 s and 120 s, when second and third aliquots were taken, respectively. For WT, a volume of 8 ml homogenization buffer was used with speed of 13,000 rpm, and time was varied from 90 s to 180 s ($n = 5$, age 70 days).

2.1.3. Staining for facilitation of counting

Samples were prepared by mixing 200 μl homogenate (from a HT in 4 ml buffer) with 0.04% Trypan blue, and physiological saline in ratios of 1:4:1, 1:1:1, or 1:1:0. All three staining ratios were prepared from the same homogenization sample ($n = 5$, age 70 days).

On the basis of outcome of the above, a standard protocol was composed and applied in the following assessments, including assessment of effects of gestational exposure to nanoparticles.

2.1.4. Variation within two halves from one testicle

The density of sperm producing tissue varies within different regions of the testes. Counts per gram testicle tissue might therefore be hypothetized to vary between the two halves of one testicle. Testicles were therefore divided into two approximately equal parts, each homogenized in 4 ml buffer and counted separately ($n = 10$, age 125 days).

2.1.5. Time span from homogenization to counting

The influence of incubation time between homogenization and counting was studied ($n = 5$, age 70 days). From each homogenate (WT in 8 ml of buffer), four aliquots of 200 μl were taken. The first aliquot was counted immediately according to the standard protocol; the second and the third were left for 4 h at room temperature and refrigerated at 4 °C, respectively. The fourth aliquot was refrigerated for 24 h at 4 °C before counting.

2.1.6. Inter- and intra-individual variation

DSP was assessed to determine inter-individual variation ($n = 10$, age 70 days). Intra-individual variation was estimated from 10 independently prepared replicates from a single homogenized testicle.

2.1.7. Variation between left and right testicles

DSP was assessed in whole left and right testicles ($n = 5$, age 210 days).