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Effects of lung exposure to carbon nanotubes on female fertility and pregnancy. A study in mice

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Highlights

- In dams, lung and liver bore evidence of MWCNT exposure 4 months after exposure
- Preconceptional exposure to MWCNT did not seem to affect pregnancy and offspring
- Delivery of a first litter was slightly delayed in female mice instilled MWCNT prior to cohabitation with a male

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Abstract

[0]We studied the effects of preconceptional exposure to multiwalled carbon nanotubes (MWCNTs): mature, female C57BL/6J mice were intratracheally instilled with 67 µg NM-400 MWCNT, and the following day co-housed with mature males, in breeding pairs. Time to delivery of the first litter, litter parameters, maternal inflammation and histopathology of lung and liver were recorded. In male offspring, locomotor activity, startle response, and daily sperm production (DSP) were assessed. In the dams, lung and liver bore evidence of MWCNT exposure when assessed 6 weeks and 4 months after exposure. A short delay in the delivery of the first litter was observed in exposed females. Litter parameters, behavior and DSP were similar in control and exposed groups. In conclusion, instillation of a single dose of MWCNT induced long lasting pathological changes in dam lung and liver. Theoretically, lung inflammation due to particle exposure could interfere with female reproductive parameters. Whether the observed lag in delivery of a first litter was in fact caused by exposure to MWCNT should be addressed in a study designed specifically to elucidate effects on the early processes involved in establishment of pregnancy. Exposure was not associated with changes in the assessed gestational or offspring parameters.

Abbreviations:

ANCOVA, analysis of covariance; ANOVA, analysis of variance; ASR, acoustic startle reaction; AVG, average of tube movements for 100 ms following onset of startle stimulus; BAL, bronchoalveolar lavage; COX, cyclooxygenase; CNT, carbon nanotube; dB(A), decibel, A-weighted; DLS, dynamic laser scattering; DSP, daily sperm production; CNT, carbon nanotube; CRP, C-reactive protein; ENP, engineered nanoparticles; EPA, Environmental Protection Agency (USA); EU, endotoxin units; ICP-OES, inductively coupled plasma-optical emission spectrometry; HE: hematoxylin and eosin; IL, interleukin; GD, gestation day; MWCNT, multiwalled carbon nanotube; NIOSH, National Institute of Occupational Safety and Health (USA); PND, postnatal day; PPI,

prepulse inhibition; R_i , optical refractive index; R_a , optical absorption index; ROS, reactive oxygen species; SAP, serum amyloid protein; SD, standard deviation; SEM, standard error of the mean; SWCNT, single walled carbon nanotube; TEM, transmission electron microscopy; TGA: thermogravimetric analysis; TNF, tumor necrosis factor; XRD, X-ray diffraction.

Keywords:

Multi Walled Carbon Nanotubes; pregnancy: developmental toxicity; reproductive toxicity; nanotoxicology; lung and liver pathology

1. Introduction

Nanomaterial research and development progress at a high pace, and engineered nanoparticles are continuously introduced to the market [1]. This highlights the need for toxicological assessment, as the risk of exposure of workers and consumers increases. Reproductive and developmental toxicity is integrated into e.g. the US EPA's nanomaterials research strategy [2] and investigation hereof is recommended by the Reproductive Health Research Team under NIOSH's National Occupational Research Agenda [3]. As yet, this area of nanotoxicology has received little attention [4;5].

Carbon nanotubes (CNTs) have attracted huge industrial interest due to their unique properties [6]. CNTs are high aspect ratio nanomaterials. Although CNTs are thinner, some types possibly have asbestos-like properties [7;8]. The airways are considered the most critical route of worker and consumer exposure [9]. As fibers, CNTs can be too long to be engulfed and removed by macrophages with very long retention times in the lungs as a consequence. A half-life approaching one year was estimated in one study of inhaled MWCNTs in rats [10]. Pulmonary exposure to CNTs induces sustained pulmonary inflammation characterized by neutrophil influx and cytokine production, fibrosis etc. [10-12].

Following pulmonary exposure to nanoparticles, inflammatory mediators are released from lung cells into circulation, potentially leading to systematic inflammation [13]. Thus, serum levels of the acute phase proteins CRP, SAP and heptoglobin and the cytokine MIP-2 were increased 24 hours after aspiration of 40 $\mu\text{g}/\text{animal}$ MWCNT and SWCNT [14;15]. Other means of systemically propagating the lung inflammatory response have recently been rendered possible. In a small controlled exposure study, healthy adult human volunteers were exposed to filtered air or diluted diesel exhaust, and RNA was isolated from peripheral blood mononuclear cells and analyzed by microarray analysis. Findings indicate transcriptional changes in inflammatory genes [16]. If activated mononuclear cells travel from the lungs into the circulation, it might have important implications for female reproductive function. During e.g. the secretory phase of the menstrual cycle, the uterine endometrium undergoes a dramatic

invasion of monocytes. These differentiate into tissue macrophages to create the inflammatory and degradative environment of menstruation. In case of implantation, menstruation is prevented, possibly due to a process of leukocyte apoptosis occurring in the vicinity of the implantation site, creating a local anti-inflammatory and implantation friendly environment [17]. Recruitment of cells primed towards an inflammatory phenotype might be hypothesized to impact adversely on reproductive processes as these generally display high sensitivity to changes in redox status [18].

Studies specifically investigating the inflammatory response in the uterus, ovaries and the fetus following lung exposure to CNTs are to our knowledge not available, but some data are available for other types of nanosized particles. Gavage of titanium dioxide nanoparticles (10 mg/kg for 90 consecutive days) thus increased ovarian ROS levels and changed the expression of several ovarian genes, but in the presence of increased levels of titanium in the ovaries [19]. Maternal airway exposure to diesel exhaust in gestation has been found to increase mRNA levels of inflammatory cytokines in the placenta, of e.g. TNF- α , IL-6, and keratinocyte-derived cytokines [20;21]. These results indicate that particulate exposures are capable of inducing inflammatory responses and possibly conditions of oxidative stress in female reproductive organs, and inflammation has been linked to adverse effects on the course of gestation and fetal development [22-25].

Inflammation may also interfere with ovulation. A series of studies in sheep elegantly show how inflammation, as a result of exposure to endotoxin, interfered with several steps in the preovulatory chain of endocrine events and thereby with ovulation. Endotoxin inhibited among others the pulsatile secretion of luteinizing hormone secretion, which provides an essential stimulus for the increase in secretion of oestradiol from the ovarian follicle preceding ovulation (overview provided in [26]). This disruption probably takes place within a sensitive time window of a few hours [27]. Interestingly, in the rat specifically IL-1 β and TNF- α , but not IL-6, inhibited the secretion of luteinizing hormone and gonadotropin releasing hormone at the level of the hypothalamus. IL-1 β and TNF- α may therefore

represent the major proinflammatory cytokines mediating suppression of the reproductive axis in inflammation [28]. Endotoxin exposure potently elevates TNF- α [28;29], and airway exposure of rodents to several types particles has also been associated with increased levels of TNF- α in lung fluid [30]. Direct effects of ENPs on central regulation of sex hormones that might indirectly interfere with reproductive processes have not been specifically investigated so far. However, polyelectrolyte multilayer coated gold nanoparticles (a potential nano-drug) have been reported to accumulate among others in the hypothalamus. Interaction of ENPs with the hypothalamus-hypothesis-gonad axes might therefore be hypothesized [4;31].

All together these data suggest that particle induced lung inflammation may interfere with female reproductive processes. Furthermore, a recent study report overt fetotoxicity after intravenous injection of different types of MWCNTs at very low dose levels [32]. The present study was initiated to investigate whether airway exposure to MWCNTs agglomerates, a potent inducer of airway inflammation, would interfere with reproductive and developmental measures. Due to the potential involvement of maternal lung inflammation in developmental toxicity, assessment of maternal inflammatory response and histopathology of lung and liver were included.

2. Materials and methods

2.1 Background for choice of study design

In a gestational exposure study (Table 1), time mated mice (n=22 per group, C57BL/6JBomTac, Taconic Europe, Ejby, Denmark) were exposed by intratracheal instillation to 67 μg four times during gestation of either NRCWE-006 (XNRI MWNT-7, Mitsui, Japan [33]) or NM-400 from the OECD Working Party on Manufactured Nanomaterials sponsorship programme [25;34] (gestational days 8, 11, 15 and 18, total dose 268 $\mu\text{g}/\text{animal}$). Controls received the 40 μl of vehicle (Nanopure water with 2% mouse serum, a pooled preparation obtained from a normal mouse population (M5905, Sigma-Aldrich)). When lung inflammation was evaluated by assessment of cellular composition in BAL

fluid, neutrophilia was obvious in the exposed females as long as 25 days after the last day of exposure. However, also eosinophilia was observed in BAL fluid in all groups at PND 2. Subsequent analysis of the mouse serum revealed a high content of endotoxin in the commercial mouse serum used for preparation of instillation vehicle. Approximately three quarters of the controls delivered a litter, compared to only half in the MWCNT groups (Table 2). No differences were observed for number of implantations and litter size, so MWCNT exposure had potentially affected early implantation in some females. Notwithstanding the endotoxin contamination, this skewing in delivering females between control and exposed females prompted us to initiate a follow-up study. The potential effects had taken place early in gestation and MWCNT-induced inflammation was furthermore hypothesized to interfere with the processes leading to ovulation. Since inflammation due to lung exposure to MWCNTs lasts for some time ([15] and present study), the mature females were exposed prior to cohabitation with a mature male, and time to delivery of a first litter was chosen as the main endpoint, as this would detect both effects on ovulation and establishment of pregnancy. NM-400 was chosen as the test material in the follow-up study, as differential cell counts of BAL fluid in the gestational exposure study indicated lung inflammation persisted longest for this MWCNT. The overall designs of the gestational and the preconception exposure studies are shown in Table 1.

2.2 Animals

Naïve mice, 60 females and 60 males (C57BL/6JBomTac, Taconic Europe, Ejby, Denmark), were supplied at 7 and 9 weeks of age, respectively. Upon arrival, the animals were distributed to cages, each holding five animals and housed in the same animal room. After one week, the females were weighed and assigned to two groups each of 30 animals, with similar weight distributions. A handful of soiled bedding from male cages was regularly added to female cages, to keep females cycling. Males were changed to single housing. Eighteen days after arrival, the females were instilled. The following day females were transferred to male cages, one female to one male. The females were weighed on a weekly basis. When body weight gain indicated conception, the male was removed from

the cage, i.e. the female was housed alone. All males were removed from the cage at termination of the cohabitation period of 8 weeks.

Animals were housed under controlled environmental conditions as described previously, with 12 hour light from 6.00 a.m. and access to food (Altromin 1324) and tap water ad libitum in polypropylene cages with bedding and enrichment (removed during nursing [35]). Procedures complied with EC Directive 86/609/EEC and Danish regulations on experiments with animals (The Danish Ministry of Justice, Animal Experiments Inspectorate, Permission 2006/561-1123). The C57BL/6J strain was used as test species, due to the widespread use of this species in nanotoxicology, including our own previous studies on developmental nanotoxicology (e.g. [25;34-37]).

2.3 Particle characterization

The NM-400 is a homogenized and sub-divided entangled MWCNTs powder prepared for the OECD Working Party on Manufactured Nanomaterials (JRC nanomaterials Repository, Ispra, Italy). Physicochemical characteristics were provided by the manufacturer [38]. In addition, NM-400 was also characterized by Transmission Electron Micrography (TEM), Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES), Thermogravimetric Analysis (TGA) and X-ray Diffraction (XRD) and Raman Spectroscopy.

For TEM, 2.56 mg of NM-400 were dispersed in distilled water containing 2% fetal bovine serum as described in [39]. The dispersions were sonicated for 16 minutes using a Vibracell™ 75041 ultrasonifier (750 W, 20 kHz, Fisher Bioblock Scientific, Aalst, Belgium) equipped with a 13 mm horn (CV33) at 40% amplitude. This setup resulted in an average horn power of about 26 W and a sample specific energy of 2614 MJ/m³. During sonication the sample vials were cooled in ice water to prevent excessive heating. After sonication, the samples were diluted with distilled water to a concentration of 0.256 mg/ml. The suspended NM were brought on pioloform- and carbon coated 400 mesh copper grids (Agar Scientific, Essex, England) that were pretreated with 1% Alcian blue (Fluka,

Buchs, Switzerland) to increase hydrophilicity as described in [40]. The electron micrographs were recorded as in [41]. The samples were imaged in bright field (BF) mode using a Tecnai Spirit TEM (FEI, Eindhoven, The Netherlands) with Biotwin lens configuration operating at 120 kV. Micrographs were recorded using a 4*4 K CCD camera (Eagle, FEI), at a magnification of 30 000 times, and a pixel size of 0.375 nm, and showed that the tubes appear as curved tubular structures (Fig. 1). The diameter was manually measured in iTEM using the arbitrary line tool, the geodesic length was measured using the polyline tool as described in [42]. The aspect ratio was calculated as the ratio of the geodesic length and the tube diameter. In representative micrographs, two operators independently measured all tubes that did not touch the borders.

For ICP-OES, approx. 50 µg of NM-400 powder were taken from the vial under gentle argon flow and weighed with an analytical balance on tinfoil to allow complete transfer into a Teflon-lined reaction vessel. An acid solution (64 ml nitric acid and 2 ml hydrogen peroxide $\geq 30\%$; suprapure quality; Sigma-Aldrich, Steinheim, Germany) was added to the vessel, sealed by a special cap (Autovent Plus) to protect the sample from excessive outer pressure during microwave digestion. The vessel was then inserted into the rotor of an Ethos 1600 microwave oven (Milestone, Shelton, CN, USA) and the selected microwave heating program was started as follows: 250 W for 5 min, 0 W for 1 min, 400 W for 5 min, and 600W for 8 min, twice, based on literature data on CNT purification processes [43;44]. After the digestion process, the reaction vessel was cooled to room temperature for approx. 2 hours and the digested sample was transferred to a Teflon flask, diluted with MilliQ water to 25 ml, and stored in Teflon flask until ICP-OES analysis (Optima 5300 DV, Perkin-Elmer, MA, USA). If any precipitate was present, the sample was discarded and the entire digestion process was repeated. Elements of concern (i.e. Al, Fe, Co, Zn, S and Si) were quantified based on external calibration curves (6 points, $R^2 > 0.995$), constructed for each element within the found concentration ranges. Signals from samples were corrected from blanks run before and after analysis. Samples were analyzed in triplicate, for three different batches.

TGA was analyzed on a Mettler Toledo TGA/SDTA 851e (Mettler-Toledo A/S, Denmark). Three samples of 8.600, 7.631 and 2.850 mg, respectively, were heated in an oxygen/nitrogen atmosphere. The heating rate was 10 K/min over a temperature range of 25 to 1000°C. The sample holders were made of alumina and had a volume of 150 μ L. The residuals from the TGA analysis were combined to a single sample and measured by XRD on a Bruker D8 Advanced diffractometer (Bruker AXS Nordic, Sweden), in reflection mode with Bragg-Brentano geometry. Sealed Cu X-ray tube run at 40 kV and 40 mA, wavelength 1.5406 Å from a primary beam Ge monochromator, fixed divergence slit 0.2°, step size 0.02, step time 1 s step⁻¹, linear PSD detector (Lynx-eye) with opening angle 3.3°. The sample was analyzed at room temperature (25°C).

The quality of the NM-400 was assessed by Raman spectroscopy, using a Horiba Jobin-Yvon Labram HR Raman spectrometer equipped with a CCD detector with a resolution of 1024 x 256. The wavelength of 632.82 nm was used to assess the intensity of the D (defect) and G (graphite) bands, which are signals reflecting the sp³- (Defect (Diamond) type) and sp²-hybridized (Graphite type) and carbon-carbon bonds, respectively.

2.4 Exposure

Mice were exposed to the MWCNTs by intratracheal instillation. Dispersions were prepared and instilled as described previously [25;34], with minor changes. MWCNTs were sonicated for 16 min (10 s pulses and 10 s pauses, total sonication time 8 min) at a concentration of 1.675 mg/mL or 1.340 mg/mL (40 μ L or 50 μ L instillation, gestational and preconceptional exposure study, respectively) in 0.2 μ m filtered, γ -irradiated Nanopure Diamond UV water (Pyrogens: <0.001EU/mL, total organic carbon: 3.0 ppb) with 2% mouse serum using a 400 W Branson Sonifier S-450D (Branson Ultrasonics Corp., Danbury, CT, USA) mounted with a disruptor horn and operated at 10% amplitude. Samples were continuously cooled by ice during the sonication procedure to prevent excessive sample heating.

The total instilled dose was 67 µg/animal. The female mice were instilled once with 50 µL of solution of vehicle with or without NM-400 followed by 150 µL of air, i.e. control and NM-400 groups, respectively. Mouse serum was prepared in our own laboratory by the following procedure. Heart blood was withdrawn from anaesthetized mature female mice (C57BL/6JBomTac, Taconic Europe, Ejby, Denmark) into Eppendorf tubes, left for 30 to 60 min at ambient temperature before centrifugation for 15 min at 2500 x g at 10°C. Centrifugation was repeated for the supernatant. Serum was kept at -80°C until use.

The stability of NM-400 dispersions was assessed by DLS and we found (data not shown) that the dispersion was satisfactory when vortexed and then sonicated in ultrasound bath for 5 min every 20 min.

The apparent particle size distribution in the particles in exposure medium was determined with a 633 nm He-Ne Dynamic Laser Scatter (DLS) Zetasizer nano ZS (Malvern Inc., UK). Data were analyzed using the Dispersion Technology Software v. 5.0 (Malvern Instruments Ltd.). Samples were measured at 25°C in 1 ml disposable polystyrene cuvettes. For calculations of hydrodynamic size, we used the optical refractive (R_i) and absorption indices (R_a) of 2.020 and 2.00, respectively for NM-400, and standard viscosity and optical properties of H₂O for the 2% serum Nanopure water vehicle.

2.5 Endotoxin content in vehicle serum and particles

A batch of 3.24 mg NM-400 was sonicated as described above in 1.0 ml pyrogen free water with 0.05% Tween 20. The suspension was then centrifuged at 25.000 x g for 6 min and the supernatant was used for endotoxin assay. The endotoxin contents were analyzed in duplicate using the kinetic Limulus Amebocyte Lysate test (Kinetic-QCL endotoxin kit, Lonza, Walkersville Inc., USA). A standard curve (ranging from 0.05 to 50 EU/ml) obtained from *Escherichia coli* O55:B5 reference endotoxin was used to determine the concentrations in terms of endotoxin units (EU) (10.0 EU = 1 ng)

in undiluted samples. In addition inhibition/enhancement controls were prepared by spiking with 10 μL of a 50 EU/ml solution.

For analysis of serum used in preparation of instillation vehicles, serum samples were diluted by a factor of four in a sterile 0.05% Tween 80 and 0.85% NaCl aqueous solution followed by 30 min heat treatment at 60°C. The samples were analyzed in duplicate for endotoxin using the kinetic Limulus Amoebocyte Lysate test as described above.

2.6 BAL preparation and analyses

BAL cell composition and neutrophil granulocyte influx were used to assess lung inflammation after pulmonary exposure to MWCNTs, 44-49 days after exposure, i.e. at the time of weaning of the first born litters (n=7 per group). BAL was collected under Hypnorm-Dormicum anaesthesia by flushing lungs three times with 1.0 mL of 0.9% sterile saline through the trachea. BAL was immediately stored on ice until BAL fluid and BAL cells were separated by centrifugation at 4°C and 400 x g for 10 min. BAL cells were resuspended in 100 μL of medium (HAMS F-12 with 10% of fetal bovine serum and 1% of penicillin and streptomycin). The number of macrophages, neutrophils, lymphocytes, eosinophils and epithelial cells were determined in 40 μL of resuspension centrifuged at 55 x g for 4 min at room temperature in a Cytocentrifuge 2 (StatSpin), by counting 200 cells fixed and stained as described in [45]. Importantly, at each time point all slides were randomized, blinded and scored by the same person. Counts are presented relative to the total cell number in the BAL fluid. The total number of live and dead cells in BAL samples was determined in further diluted suspension (20 μL of cells in 180 μL of HAMS F12 medium with FBS and PS) by counting in a NucleoCounter, following the standard kit procedure (Chemometec, Denmark).

2.7 Maternal lung and liver histology

Dams were anaesthetized by subcutaneous injection of Hypnorm-Dormicum and killed by withdrawal of heart blood, 6-7 weeks (i.e. after accomplished pregnancy and lactation, and at a time where the offspring were independent of maternal care) or 4 months after the single exposure to 67 µg of NM-400, to assess the effects of MWCNT exposure on a longer term (n=3 and 6 per group, respectively). The lungs were fixed in situ, by cannulating the trachea and delivering 4% neutral buffered formaldehyde via the cannula at a constant fluid pressure of 25 cm before the thorax was opened [46]. Thereafter the lungs were excised and immersed in 4% neutral buffered formaldehyde solution until further processing. Livers were excised and specimens were fixed in the formaldehyde solution. The tissues were then embedded in paraffin, sectioned in 4-6 µm slices and stained with hematoxylin and eosin for histological examination.

2.8 Parturition and lactation

Females were weighed on a weekly basis after start of cohabitation. All cages were checked at least once daily at 7 a.m. for delivery, and the day of delivery was noted. On PND 3, offspring were counted and sexed, and dams with pups were weighed individually at PND 3, 10, and 21. A maximum of one male pup per litter were weaned for assessment of sperm counts in adulthood. The same pups were used in behavioral tests (14 controls and 12 NM-400s). Weaning was performed in 3 sets, with animals from control and exposed groups matched according to day of delivery after start of cohabitation. This ensured that the estimation of endpoints had a similar distribution in the two exposure groups relative to day of birth and time after exposure, for both dams and offspring.

During the cohabitation period, one control female was supplied with a new male partner within a few days, and two control females had to be excluded from the study due to violent encounters with their male partner. One control female delivered a litter two days after termination of the 8-week cohabitation period. In the exposed group, three females were excluded. One female had to be killed due to pregnancy complications, and one female displayed weight gain indicative of pregnancy, the male was removed, but no birth was observed. At termination of the study, she had one uterine

implantation scar. For a third female, the male partner was removed also as indicated by weight gain, but without the female having conceived.

2.9 Behavioral testing

Male offspring was tested for reactivity in the acoustic startle test and for locomotor activity in the open field (one male per litter, n=12-14). Investigations were performed during the light period, and exposed and control animals were tested alternately. Animals were transferred to the experimental room 1 hour before the first test.

Acoustic startle reaction (ASR) and prepulse inhibition (PPI) were tested one week after weaning (4 weeks of age) as described [47] in two chambers (San Diego Instruments, San Diego, USA) with 70 dB(A) white background noise. A piezoelectric accelerometer transduced displacement of test tubes (\varnothing 3.6 cm) in response to movements of the animal. Animals were acclimatized for 5 min in the tube before sessions started and ended with 5 startle trials of 40 ms 120 dB(A) bursts of white noise. In between, 35 trials were delivered in semi-randomized order (10 trials of 120 dB(A); 5 each of 4 prepulse + startle trials (prepulses of 72, 74, 78, and 86 dB(A)); 5 trials with only background noise). Tube movements were averaged over 100 ms following onset of the startle stimulus (AVG). The five AVGs for each prepulse intensity were averaged and used to calculate PPI, which was expressed as percent reduction in AVG compared to the average of the 10 middle startle trials: $\%PPI = 100 - ((AVG \text{ at prepulse+startle trial}) / (AVG \text{ at startle trial})) * 100\%$.

Activity level was assessed for 3 min at 13-14 weeks of age in a circular, white open field (\varnothing 100 cm) as described [36] with minor modifications. Trials commenced in the center of the field and the location of the animal was registered by Noldus Ethovision (Version 5, Noldus Information Technology, Wageningen, Netherlands). The tracking device calculated total ambulation, which was subsequently split into three time-bins of 1 min to test for habituation. Duration in the central and the

outer 9 cm peripheral zone of the field, as well as the number of crossings from the outer to the central zone were extracted.

2.10 Sperm production

At the age of 125 days, male offspring were killed and testes excised (n=12-14), placed in NUNC cryotubes, snap frozen in liquid Nitrogen and stored at -80°C until assessment of daily sperm production (DSP) as described in [37]. In short, the left testes were decapsulated and cut into halves (approximately 50 mg tissue each). Each half was weighed and homogenized by IKA ULTRA TURRAX® T25 (disperser S25N-10G) in 4 ml of 0.05% TRITON-X-100 homogenization buffer for 90 sec at 13 000 rpm. Homogenates were stained by 0.04% Trypan blue (Hopkin and Williams, in 1x phosphate buffer), and homogenization resistant elongated spermatids (stage 14 – 16) were counted using a haemocytometer (Bürker chamber, 0.0025 mm², depth 0.100 mm) under light microscope at 400x magnification. The average value of the two halves from each testis served as the basis for calculation of the number of spermatids per g testis tissue and the total number of spermatids produced per day in the left testis (DSP). DSP was calculated by dividing the total number of homogenization resistant elongated spermatids in testes with the time divisor value of 4.84. This number corresponds to the time (in days) in which developing spermatids spend in stage 14 – 16 during spermatogenesis in the mouse.

2.11 Statistics

Litter was considered the statistical unit. Due to accidental high content of endotoxin in mouse serum used for preparation of instillation vehicle, data from the gestational exposure study are described briefly but only subjected to statistical analysis in a few instances for hypothesis generating purposes (gestation length and littering success were thus analyzed by Fisher's exact test). In the preconceptional exposure study, gestational parameters were analyzed by Mann-Whitney *U*-test, and time to delivery of a first litter by log rank test. ANOVA was applied to remaining data, when relevant with repeated measures in trials, days, or time-bins. ANCOVA controlled for litter size in both overall

and pair wise comparisons, birth weights, and pre-weaning pup weights. PPI was analyzed separately for each prepulse intensity [47]. Analyses were performed in SYSTAT Software Package 9 and SAS 9.2.

3. Results

3.1 Particle and exposure characteristics

Primary and exposure characteristics of NM-400 are presented in Fig. 1 and Table 3. TEM showed that the NM-400 consisted of sub- μm long and highly curved MWCNTs, with a mean diameter of 10 nm (Fig. 1A and Table 3). The diameters of all the CNTs were smaller than 100 nm with relatively low overall variation (Fig. 1B). The geodesic length (Fig. 1D) and corresponding aspect ratios, however, varied greatly, but were shorter than reported by the manufacturer. It is likely that the tube-length was shortened during the probe sonication [48], explaining at least part of the discrepancy between our findings and information from the manufacturer, c.f. Table 3.

[0]

TGA showed that acceptable sample homogeneity was obtained in samples of 10-15 mg. At these sample sizes, NM400 contained 16 ± 3 wt% of incombustible impurities (buoyancy not taken into account). TGA also showed that the sample decomposed in several steps indicating the presence of several different carbon compounds or MWCNT qualities.

XRD analyses of the residual material after TGA analysis showed a few very broad reflections, indicating that it contained crystals smaller than 10 nm, tentatively identified as γ -alumina or alternatively θ - Al_2O_3 or a mixed oxide alumina, e.g. ($\text{Mg}_{0.866}\text{Al}_{1.8303}\text{O}_{3.661}$). The alumina most likely arises from the support material for the catalyst used in the production of the MWCNTs.

ICP-OES analysis showed that the catalyst content is rather important with aluminum (Al, 5.3 wt %), iron (Fe, 0.4 wt %) and cobalt (Co, 0.2 wt %) being the dominant impurities detected. XRD of the residual after the TGA analysis supported the presence of an alumina oxide, as did also TEM where

crystalline electron dense impurities were observed. Both TGA and the ICP-OES analysis of Na indicated that the sample was inhomogeneous with respect to this element (Table 3).

Results from Raman spectroscopy of NM400 showed high intensity of the D-(Defect)-band at ca. 1290 cm^{-1} , which clearly exceeded the G-(Graphite)-band at ca. 1582 cm^{-1} . The G' band at about 2700 cm^{-1} is an overtone of the “regular” D-band and was also clearly observed. For high quality CNTs the D/G ratio is only a few percent, but the ratio increases with increasing number of walls in MWCNTs. However, considering that the average diameter of the MWCNTs is only 10 nm in this NM400, the observed D/G ratio indicates high abundance of structural defects in these MWCNTs.

DLS showed an average zeta-size of approximately 89 nm and the apparent hydrodynamic number size-distributions had peak size at 51 nm (Fig. 2). Even though, the hydrodynamic equivalent spherical size is difficult to interpret for DLS, the data and dispersion stability suggest a reasonable de-agglomeration by the applied dispersion protocol.

3.2 Endotoxin content in vehicle serum and particles

Endotoxin concentration was below the detection limit of 0.05 EU/ml for NM-400. In the commercial serum used for exposure in the gestational exposure study, the concentration was 6557 EU/ml of serum, corresponding to a total of 21.0 EU/mouse for the four instillations. In the serum used for preconceptional exposure, the concentration was 0.93 EU/ml of serum or 0.00074 EU/mouse for the single instillation.

3.3 Lung inflammation in mated females

Lung inflammation was evaluated by the cellular composition in BAL fluid 6-7 weeks after exposure. There were no significant differences in BAL cell composition at about six weeks after the single instillation of NM-400 MWCNT, apart from relatively more dead cells in BAL from exposed females [$F=(1,11)=6.945$, $p=0.023$, ANOVA].

3.4 Maternal lung and liver histology

The results from the histopathological examination of lungs and livers from dams exposed to a single dose of 67 μg of MWCNT NM-400 are summarized in Table 4. Six weeks after exposure, two out of three MWCNT mice displayed histopathological changes in the lungs. Bronchiolar subepithelial edema was observed in two of three exposed animals but in none of the controls (incidence: 2/3 vs. 0/3 in the controls), as was perivascular edema of connective tissue (incidence: 1/3 vs. 0/3) and hyperplasia of bronchiolar epithelial cells (incidence: 2/3 vs. 0/3) in the lung (Fig. 3). Apart from these lesions single macrophages occurred sporadically (incidence: 2/3 vs. 0/3). After four months, changes in lung included infiltration of mononuclear cells close to bronchiolar and arterial walls (incidence: 6/6 vs. 0/6), and of macrophages (incidence: 3/6 vs. 0/6). Macrophages were mostly observed as aggregates of several cells (Fig. 3 G). Edema of supportive tissue surrounding bronchioles and/or blood vessels was also seen (incidence 2/6 vs. 0/6). Desquamation of the bronchiolar epithelium was recorded in the lungs from one exposed (1/6) and two control dams (2/6).

In the livers from exposed dams, modest histopathological changes were recorded (Fig. 4). The lesions recorded 6 weeks after exposure included: microfoci of necrosis (incidence: 3/3 vs. 1/3 in the controls), enlargement of single hepatocytes (incidence: 2/3 vs. 0/3), increased number of Kupffer cells and binucleate hepatocytes in exposed compared to control livers (incidence 3/3 vs. 0/3). Four months after exposure microscopic changes included focal hyperplasia and hypertrophy of Kupffer cells (incidence: 3/6 and 4/6, respectively, vs. 0/6 in controls). Furthermore, foci of inflammatory cells (incidence: 1/6 vs. 0/6) and edematous endothelial cells (incidence: 1/6 vs. 0/6) were observed. Microfoci of necrosis were seen in some livers from exposed (4/6) as well as control (3/6) animals.

3.5 Parturition, time to delivery of a first litter and lactation

Mature, naïve females were exposed once to 67 μg of NM-400 by instillation and the following day initiated cohabitation with unexposed, mature male mice. During the 8 week observation period, the

control and exposed groups delivered comparable numbers of litters, i.e. 26 of 28 controls and 24 of 27 exposed females delivered offspring. For exposed females, delivery of the first litter was delayed on average 5 days ($p < 0.01$, log rank test). The difference remained statistically significant after exclusion of females with post-instillation weight loss ($p < 0.05$, log rank test, Fig. 5. Mean \pm SEM: Controls: 22.12 ± 0.65 days; MWCNT: 26.72 ± 1.84 days). Reanalysis with inclusion of otherwise excluded conceiving females had no effect (as described in the method's section for parturition and lactation). Gestational and litter parameters were similar in control and exposed groups (Table 2).

3.6 Offspring behavior and sperm production

Activity in the open field test and the acoustic startle response was determined for 4 or 13-14 week old male offspring, respectively. No differences were observed (data not shown). Daily sperm production and testis size in male offspring at age 125 days were unaffected by the maternal exposure (data not shown).

4. Discussion

The present study examined effects on the establishment of pregnancy and the course of gestation following a single instillation of $67 \mu\text{g}$ of NM-400 one day prior to cohabitation with a mature male. Litter size and birth weights were comparable across groups, as were behavior and DSP in the offspring. A delay in delivery of the first litter was observed in the exposed females compared to controls.

When lung inflammation was assessed six weeks after pulmonary deposition of the single dose of NM-400, the cell profile in BAL fluid from exposed females was similar to that of controls, apart from an increased number of dead cells. It is well established that exposure to MWCNTs induces a dose-related and robust inflammatory lung response shortly after exposure as evidenced by e.g. influx of neutrophil granulocytes and increased levels of inflammatory cytokines in BAL fluid. The response resolves over time, and e.g. neutrophil numbers diminish over time even if MWCNTs are still present

in the lung, although this is highly dependent on the dose [10;11]. Overall, the exposed females would be expected to have experienced a marked inflammatory response shortly after instillation followed by a gradual reduction, in agreement with the similar neutrophil cell numbers observed in control and exposed dams 6-7 weeks post exposure. This is substantiated by the histopathological observations. Accordingly, the changes observed in the lungs six weeks after exposure correspond to the expected response to injury after deposition of particles in the airways [49]. Four months after exposure to NM-400, mononuclear infiltration was still apparent in the lungs of all exposed females, and conglomerates of macrophages contained what possibly was indigestible test material in accordance with observations reported for MWCNT exposed rats [50]. Similarly, exposure of rats to SWCNT was reported to be associated with alveolar macrophage accumulation [51]. Such changes are a recognized part of the lung response to injury, but the further fate of the phagocytized MWCNTs remains to be elucidated. After a single administration of particles to the lung, the amount of particulates in the lung is expected to decrease. In the alveolar region, clearing mechanisms include macrophages, interstitial pathways and dissolution. Digestible material phagocytized by the macrophages is either destroyed or solubilized and absorbed into the circulation. Macrophages containing indigestible material may also be cleared by the mucociliary escalator, enter the lymphatic system and be deposited in lymph nodes, be retained within the lung or even destroyed by the ingested material [49]. It has not been convincingly demonstrated that MWCNTs can translocate from the lungs to the circulation. However, in the liver we observed an increased number of Kupffer cells in all exposed dams after six weeks, as well as significant hypertrophy and hyperplasia of these cells after four months. Thus it can be speculated whether MWCNTs entered the liver via the circulation and were taken up by Kupffer cells in accordance with their role in the mononuclear phagocyte system. On the other hand, the pulmonary inflammation and resulting circulating cytokines may have systemic effects on liver. An increased number of binucleate hepatocytes and enlarged hepatocytes six weeks after the exposure can be indicative of a regenerative activity in the liver [52]. Microfoci of necrosis and inflammatory cell foci are known to occur also under physiological conditions in untreated animals [53], but the increased incidences of these histopathological changes in the present study indicate their relation to treatment.

Such lesions have previously been reported in rats exposed by inhalation to silver nanoparticles [54;55], although no liver changes were reported in rats exposed to MWCNTs by intratracheal instillation [50;51] or by inhalation [10;11].

Several possible mechanistic pathways may influence reproductive and developmental processes. Delayed time to delivery of a first litter is an apical endpoint, reflecting (cumulative) decrements in one or several subordinate processes and endpoints, e.g. altered mating behavior, impaired ovulation or cyclic arrest, insufficient preparation of the uterine lining, delayed blastocyst implantation, decreased survival of the early conceptus, failure to establish maternal-fetal circulation, etc. [56;57]. Nanomaterials have been suggested to affect reproduction and development by direct as well as indirect means. If translocated to the target organ, nanomaterials might affect tissue or interfere with physiological processes, either due to the physical presence of particles, through generation of reactive oxygen species (ROS) or due to toxicity related to chemical constituents of the particles (e.g. core or other associated compounds). As for indirect pathways, airway exposure to nanoparticles induces acute phase response, inflammation and oxidative stress in pulmonary tissues, initiating release of cytokines, acute phase proteins and other signaling molecules with potential adverse effect for reproductive processes (summarized in [4;5;13]).

We have not located studies of distribution of CNTs to female reproductive organs, but intravenously injected carbon nanotubes have been shown to migrate to the testes in rats [58]. Other ENPs have been observed to reach the female reproductive organs, even following airway exposure. When female mice were exposed by nose-only inhalation to 50 nm iron-containing ENPs (4 hours/day, 5 days/week for four weeks), particles were observed in various organs, including the ovaries [59]. After intraperitoneal administration of 50 nm magnetic particles (25-100 mg/kg) particles were observed in the uteri of mice [60]. In vitro, ovarian granulosa cells have been shown to respond to nanosized particles of gold and calcium phosphate [61;62], indicating particles possess at least potential for interfering with ovarian processes. Physical presence of particles does not necessarily lead to adverse

effects in differentiating tissues. Silica nanoparticles of 10 and 30 nm inhibited differentiation of embryonic stem cells into spontaneously contracting cardiomyocytes. In contrast, particles of 80 and 400 nm did not cause this effect, even if the largest particles were clearly visible inside vacuoles of the embryoid bodies [63]. Overall, direct particle effects on female reproductive tissue and function cannot be excluded. Whether MWCNTs translocated from the lungs to the reproductive organs in the abdominal cavity within the period of a few days is however questionable [64].

Apart from carbonaceous material, NM-400 contained elemental impurities. The total impurity level was in the order of 16 wt%, among others consisting of Al (5.3 wt %), Fe (0.4 wt %) and Co (0.2 wt %). Al, Fe and Co have all been associated with adverse effects on female reproduction [65-69], but at much higher dose levels than the total doses of 0.14, 0.011 and 0.005 mg/kg of Al, Fe and Co, respectively, in the present study. At these dose levels, it is unlikely that these elemental impurities affected female reproductive function. The presence of these metals in the lungs may very likely have contributed significantly to an inflammatory response [69;70].

The delay in delivery of the first litter in NM-400 exposed dams roughly corresponded to one estrous cycle. We have not been able to identify other studies of airway exposure to CNTs and establishment of pregnancy. One study investigated reproductive capacity in mice housed in outdoor exposure chambers in an urban area heavily polluted by traffic generated particles. Living in unfiltered chambers was associated with an increased number of days in estrus, and therefore a prolonged total estrus cycle. Further, the time necessary for mating to occur was more than doubled. Breeding couples living under ambient air conditions showed impaired fertility and pregnancy parameters when compared to couples living under filtered air conditions. These latter outcomes may however be ascribed to male as well as female factors [71]. Since female rodents will not engage in mating until receptive [56], the prolonged time to mating might occur due to prolongation of the estrus cycle. As described, lung exposure to particles is a potent inducer of inflammation. Potentially, particle induced

inflammation may have induced behavioral changes resulting in a delay of copulation [72]. As described in the introduction, inflammation is also a known inhibitor of ovulation.

To our knowledge, the present study is the first to address the effects of lung exposure to CNTs on female reproductive events and of periconceptional exposure to engineered nanoparticles in general. Several limitations in the study design have to be considered, though. Estrous cyclicity prior to dosing was not assessed. Such data would have provided information as to whether a greater percentage of dams in the dosed versus the control groups were in diestrus when cohoused with the undosed male. To keep females cycling, the female cages were regularly supplied with soiled bedding from male cages and males were housed in the same room during the acclimation period preceding dosing. Furthermore, (unpublished) data of cyclicity from C57BL/6J mice received and housed under the same conditions as in the present study do not indicate differences between arbitrary groups of females. Vaginal smearing post-exposure would further have allowed for some characterization of the cause of the delay, e.g. determination of mating performance, timing of copulation and pre-coital interval, failure to conceive, and gestation length. As a similar percentage of females delivered a litter during the follow-up period, exposure to MWCNTs was not associated with an overall failure to conceive. As is, the data obtained from the present study design do not allow for elucidation of the biological mechanism underlying the delay of delivery of the first litter.

Unfortunately, in the gestational exposure study the serum used for preparation of instillation vehicle was contaminated with endotoxin. Endotoxin is composed of lipopolysaccharides, a non-allergenic cell wall component of gram-negative bacteria with strong pro-inflammatory properties.

Approximately 0.5 ng endotoxin was instilled in the present study. In studies of endotoxin mediated inflammation 10-100 ug endotoxin are routinely used, and 100 ng is considered a low endotoxin dose, which elicits a limited inflammatory response [73]. The concentration of endotoxin in the serum sample was at a similar level as in mouse serum in the case of endotoxin shock [74]. The used serum was therefore most probably contaminated post harvesting rather than originating from infected animals. The accidental endotoxin exposure in the present study emphasizes the importance of

enforcing appropriate control measures, even when using commercial products. The incident prompted us to produce our own serum for future studies, and to routinely measure endotoxin content in vehicle and in nanoparticles [13;75]. Particles as well as our own serum displayed very low levels of endotoxin, that would not be expected to affect the animals [76].

The applied study design does not allow for firm conclusions regarding female lung exposure to particles and potential interference with female reproductive parameters. The observation of a slight delay in delivery of a first litter must necessarily be hypothesis generating. Generally, transient elevations in inflammatory or oxidative status might be restored within relatively short time. In reproductive and developmental physiology even a fleeting inflammatory or oxidative peak might lead to definitive events if occurring during a sensitive time window, as e.g. described above for ovulation. Furthermore, most reproductive and developmental processes require a certain amount of oxidative stress to proceed, and reactive oxygen species even serve as key signal molecules in physiological processes. At the same time, imbalance between oxidative and antioxidative processes might prove detrimental, and reactive oxygen species can be considered cofactors in pathological processes. Finally, the antioxidative defense is naturally weak during several crucial reproductive and developmental events, e.g. during the shift from reductive to oxidative nutrition of the fetus {Al-Gubory, 2010 8638 /id;Rizzo, 2012 8636 /id;Dennerly, 2010 8637 /id}. In conclusion, instillation of a single dose of MWCNT induced pathological changes in lungs and livers as long as 4 months after exposure. In addition, a slight delay in the delivery of a first litter was observed, but exposure did not interfere with behavior and sperm quality in male offspring, even though exposed females would be expected to have experienced lung inflammation during gestation. Theoretically, lung inflammation due to particle exposure could interfere with female reproductive parameters. Future studies should aim to address the underlying biological mechanism.

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Captures to figures

Fig. 1. Illustration of the MWCNT nanomaterial NM-400. Representative TEM image (A) showing the MWCNT to be long and highly curved. Bar = 100 nm. Fibers were manually measured to give number based thickness (B), number based aspect ratio (C) and number based geodesic length (D) distributions.

Fig. 2. Hydrodynamic particle size distribution of intratracheally instilled NM-400 dispersions as measured by Dynamic Light Scattering. Hydrodynamic number size-distributions peaked at 51 nm.

Fig. 3. Lung morphology. Microscopic pattern of the mouse lung: A, B: Normal structure in vehicle exposed control animals. A: 6 weeks; B: 4 months. C, D: Pathological findings in MWCNT exposed females after 6 weeks: Subepithelial edema of the bronchiole, under the wall of the bronchiole and perivascular edema close to the vein wall (asterisks); hyperplasia of the epithelial cells (thin arrow); congestion (thick arrow). E – H: Pathological findings in MWCNT exposed females after 4 months: Macrophages (long arrows); desquamation of the bronchiolar epithelium (short arrow); edema under the wall of the bronchiole (asterisks); infiltration of mononuclear cells close to arterial and bronchiole wall (thick arrow). G: Pattern of aggregations of macrophages – main figure has scale as in figure H, insert is magnified by 1.8 x. Staining HE, magnification as in figure H.

Fig. 4. Liver morphology. Microscopic pattern of the mouse liver: A, B: Normal structure in vehicle exposed control animals. A: 6 weeks, B: 4 months. C, D: Pathological findings in MWCNT exposed females after 6 weeks: Microfoci of necrosis (N); enlargement of single hepatocytes (long thin arrows); increased number of binucleate hepatocytes (short thick arrows); increased number of Kupffer cells (short thin arrows). E, F: Pathological findings in MWCNT exposed females after 4 months: microfoci of necrosis (N); focus of inflammatory cells (short thick arrow); hypertrophy and hyperplasia of Kupffer cells (short thin arrows); edematous endothelial cells in central vein (long arrows). Staining HE, magnification as scale on figure H.

Fig. 5. Littering curves for mature female mice exposed by intratracheal instillation to vehicle or to MWCNT NM-400 the day before cohabitation with a male.

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Table 1: Overall design of the gestational and preconception exposure studies

	Gestational exposure		Preconceptional exposure	
Particles	NRCWE-006 and NM-400		NM-400	
Initial group size	22		30	
Time of exposure	GD 8, 11, 15 and 18		One day pre-mating	
Dose	4 x 67 µg/animal (total 268 µg)		67 µg/animal	
Endpoints	Dams	Offspring	Dams	Offspring
Gestation and litter parameters	+	+	+#	+
Time to delivery of a first litter			+	
Lactational weight gain	+	+	+	+
BAL count	NP: 1 (3) P: 24±1 (26±1)		P: 23±2 (46±2)	
Histology			42±2 120±2	
Behavior:				
- Acoustic startle				28±2
- Open field				95±2
DSP				125

Assessment of a given endpoint is indicated by a + or a number, the latter indicating the time of testing in postnatal day. Number in parentheses refers to days after exposure. GD: Gestational day. PND: Postnatal day. NP: Nonpregnant time-mated females. P: Littering females. *Maternal weight gain and gestation length not monitored.

Table 2. Gestational and lactational data for littering females.

	Gestational exposure			Preconceptional exposure	
	Control	NM-400	MWCNT-7	Control	NM-400
No. females	22	21 [§]	22	28 [#]	27 [#]
Litters (%) ^{##}	72.7	57.1	45.5	92.9	88.9
Maternal weight gain, GD8-18 (g)*	9.0±0.5 ^a	8.2±0.6 ^(a)	6.8±0.5 ^b	-	-
Pups/litter PND2/3	5.94±0.4	5.17±0.5	5.10±0.7	6.11±0.4	5.79±0.4
Implantations ^{###}	7.69±0.5	8.22±0.7	7.44±0.5	7.70±0.4	6.84±0.5
Implantation and perinatal loss (%)	25.8±3.0	40.4±9.1	38.5±5.9	19.2±2.9	20.4±3.0
Gestation length*	20.0±0.0 ^a	20.0±0.0 ^a	20.5±0.2 ^b	-	-
Pup body weight:					
PND 2/3 (g)	1.33±0.02	1.27±0.03	1.31±0.03	1.46±0.02	1.54±0.03
PND 8/10 (g)	4.35±0.12	4.13±0.16	3.84±0.25	5.28±0.11	5.49±0.11
PND 22/21 (g)	7.51±0.37	7.73±0.35	7.72±0.41	8.64±0.21	8.48±0.40
Sex ratio (%fem)	57.7±8.1	45.4±7.2	68.9±5.6	59.3±4.7	56.0±5.0

Data are given as mean ± SEM. For the gestational exposure study, the overall study protocol varies slightly from protocol described for the preconceptional exposure study. [§]One female died due to failed instillation. [#]Study population initially included 30 females/group. Two control and one CNT female had to be killed due to complications unrelated to exposure, and from two CNT females the males were removed before conception due to increased weight gain but either no pregnancy or birth took place without noticing. ^{##}For preconceptionally exposed, within 8 weeks from start of cohabitation. ^{###}Data were accidentally lost from 7 females in the preconceptional exposure study, equally distributed between groups. Values in the same row not showing a common superscript (a, b) differ statistically significantly. *P<0.05.

Table 3. Key physico-chemical characteristics of NM-400

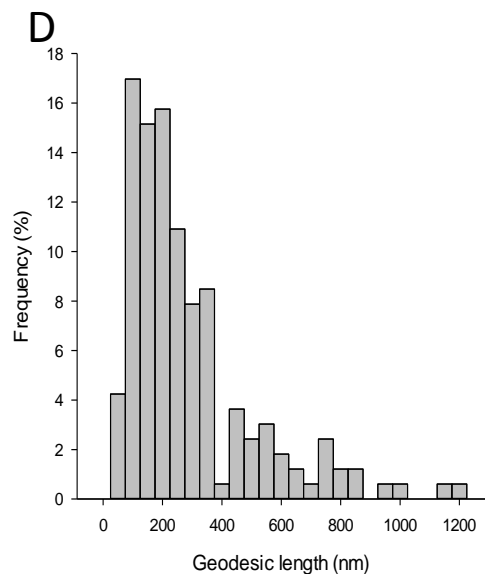
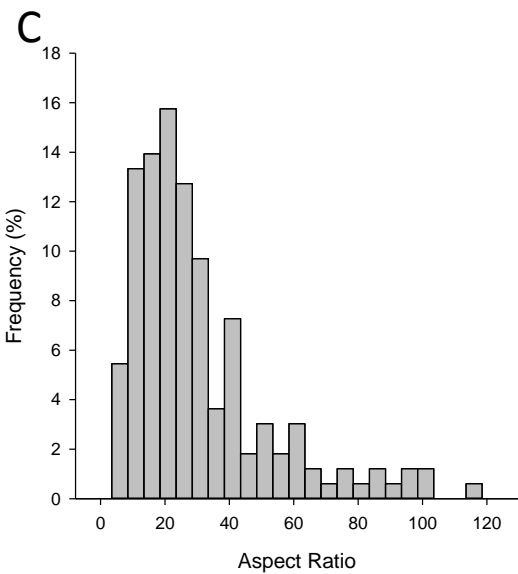
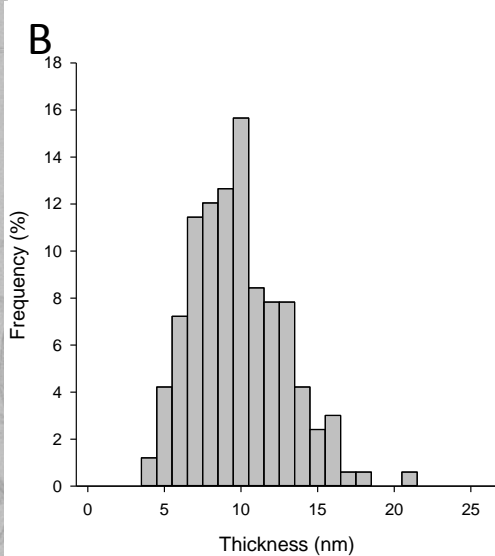
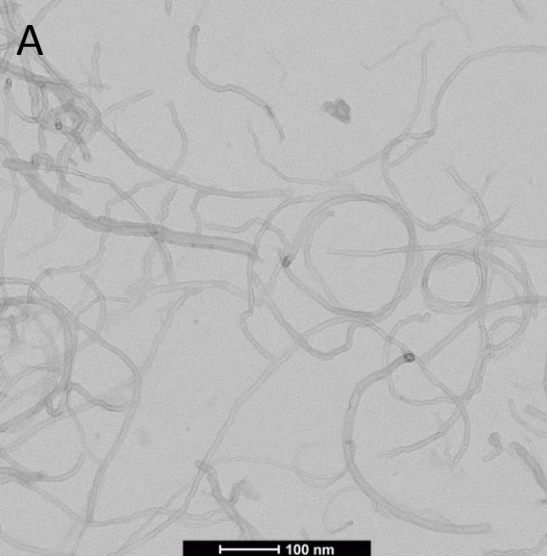
Particle characteristics	Manufacturer	Ref. (78)	This study
Particle size			
Length	~ 1.5 μm	0.7 - 3 μm	295 \pm 234 nm (geodesic)
Diameter	~ 9.5 nm	5 – 30 nm	10 \pm 3 nm
Aspect ratio			31 \pm 26
Phase	MWCNT	Entangled kinked MWCNT 10 – 20 walls	Highly bent MWCNT
Surface area (BET)	250-300 m^2/g	298 \pm 1 m^2/g	
Chemical composition	10 wt% Oxides/coated with pyrogenic carbon		Al: 53447 \pm 136 ppm Fe: 3658 \pm 31 ppm Co: 2067 \pm 7 ppm Na: below MDT (10 ppm), but higher concentrations unevenly distributed
Thermogravimetric analysis	90 % C		Weight loss 84 \pm 3 wt%*. Inhomogeneous sample, representative subsample > 8 mg (O_2 atmosphere, 30-800 $^\circ\text{C}$, 10 $^\circ\text{C}/\text{min}$)
XRD (TGA residual)			Mainly amorphous, however indications of alumina oxide

* Buoyancy not taken into account

Table 4. Type and incidence of histopathological changes in lungs and livers from mice 6 weeks or 4 months after a single intratracheal instillation of 67 µg MWCNT NM-400.

Organ	Type of lesion/time post treatment	Control	MWCTN
Lungs			
<u>6 weeks</u>			
	Subepithelial edema of bronchiole	0/3	2/3
	Perivascular edema	0/3	1/3
	Hyperplasia of epithelial cells of bronchioles	0/3	2/3
	Single macrophages	0/3	2/3
<u>4 months</u>			
	Desquamation of bronchiole epithelium	2/6	1/6
	Edema of supportive tissue of bronchiole and/or blood vessels	0/6	2/6
	Infiltration of mononuclear cells	0/6	6/6
	Aggregates of macrophages	0/6	3/6
Liver			
<u>6 weeks</u>			
	Microfoci of necrosis	1/3	3/3
	Enlargement of single hepatocytes	0/3	2/3
	Increased number of binucleate hepatocytes ^a	0/3	3/3
	Increased number of Kupffer cells ^a	0/3	3/3
<u>4 months</u>			
	Microfoci of necrosis	3/6	4/6
	Edematous endothelial cells	0/6	1/6
	Inflammatory cell focus	0/6	1/6
	Hyperplasia of Kupffer cells	0/6	3/6
	Hypertrophy of Kupffer cells	0/6	4/6

^a Qualitative evaluation only



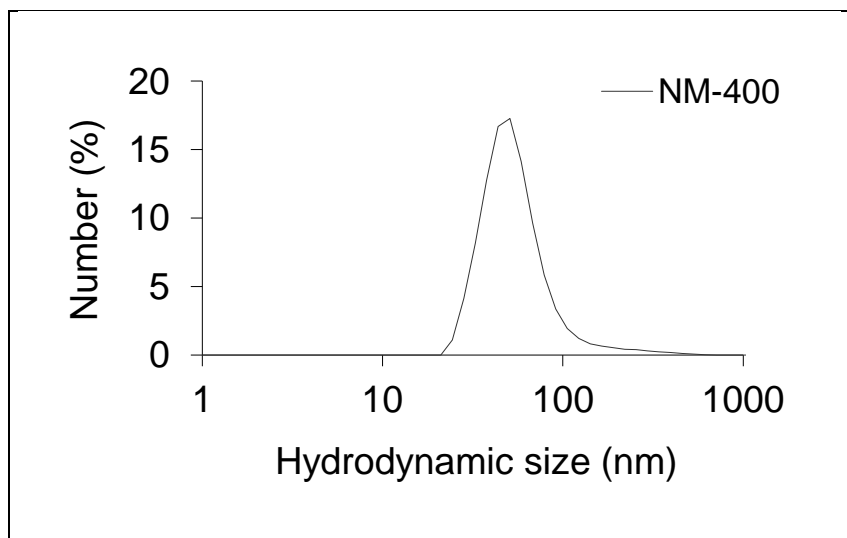
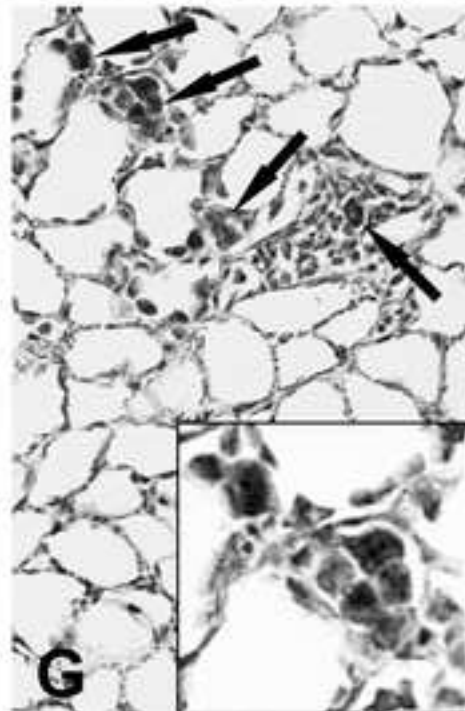
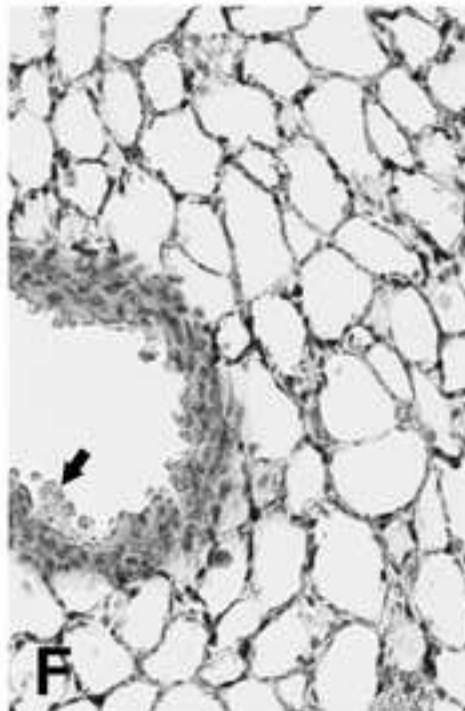
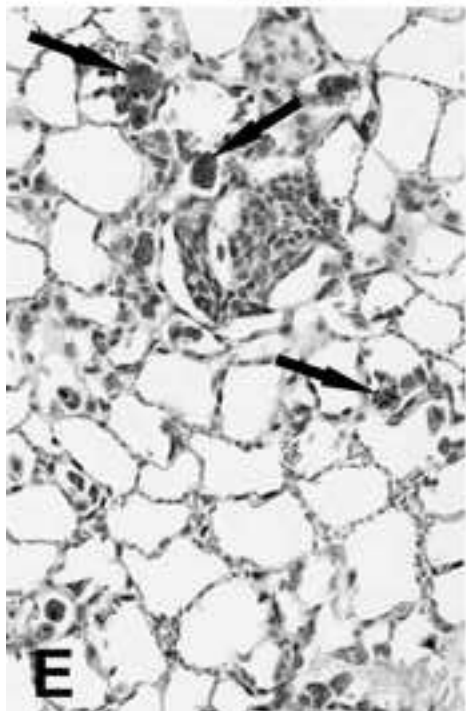
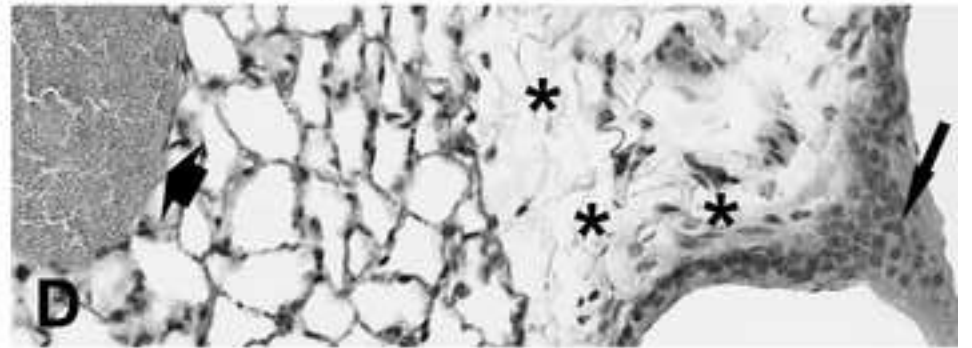
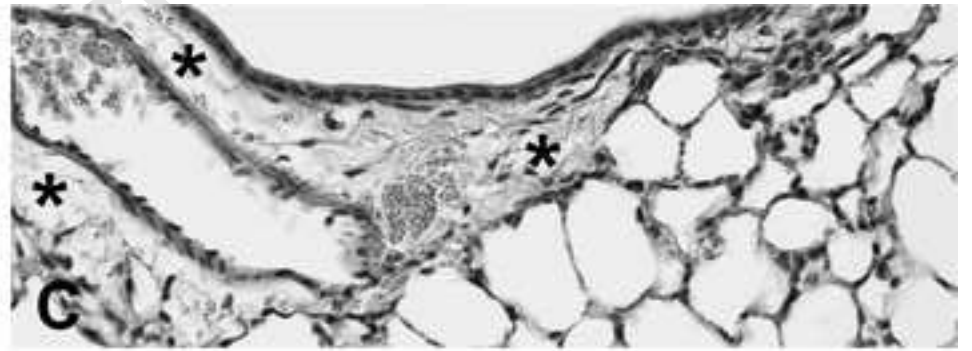
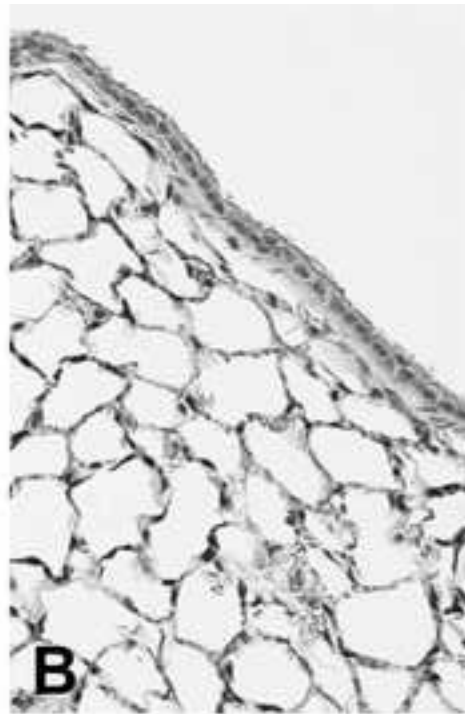
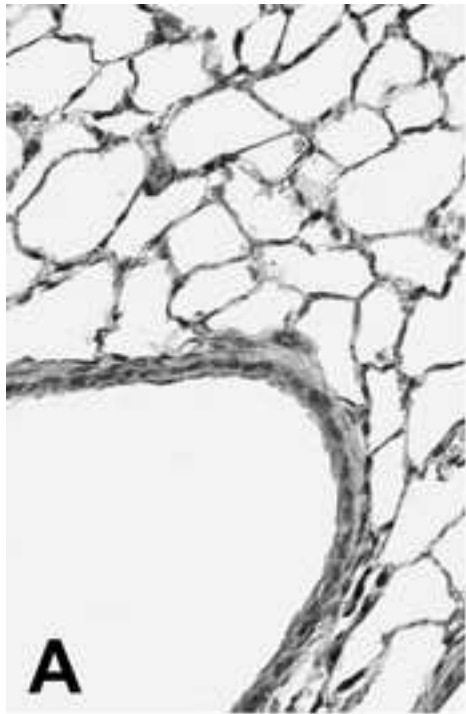
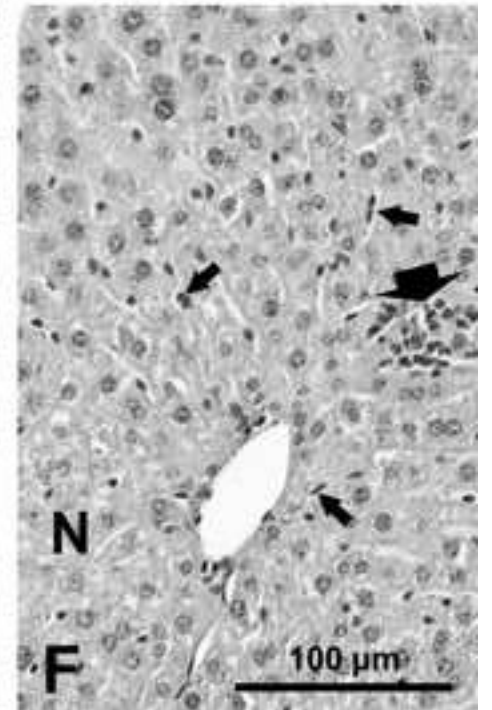
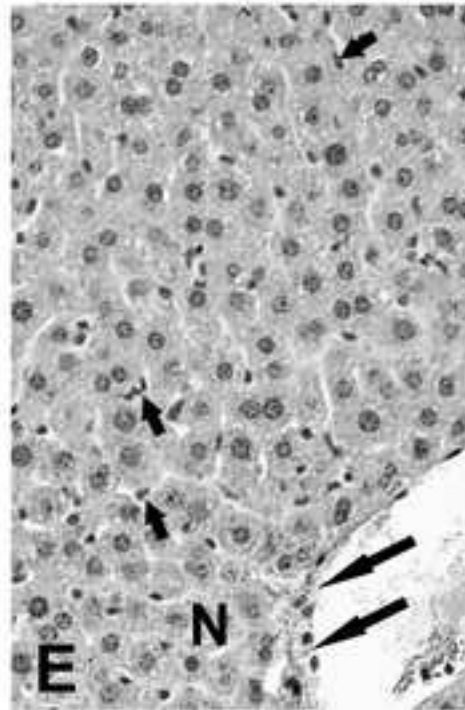
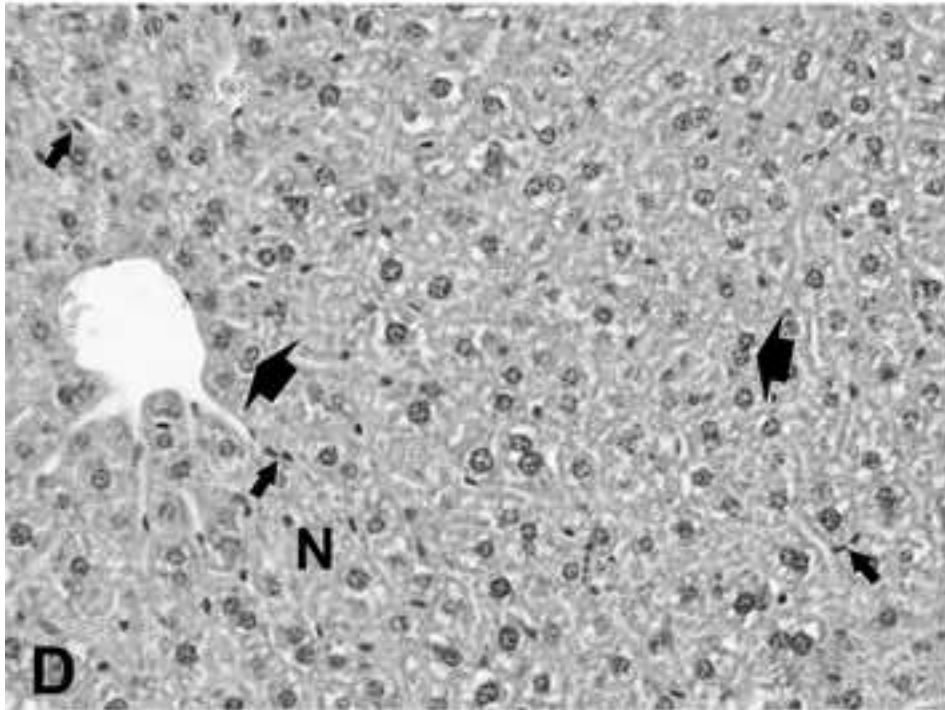
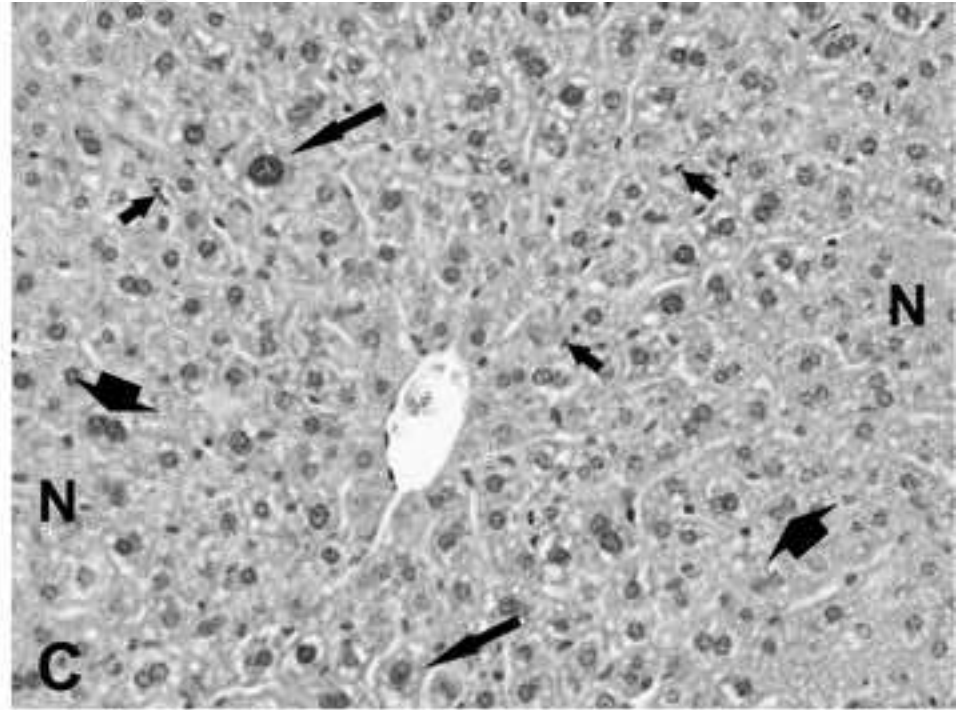
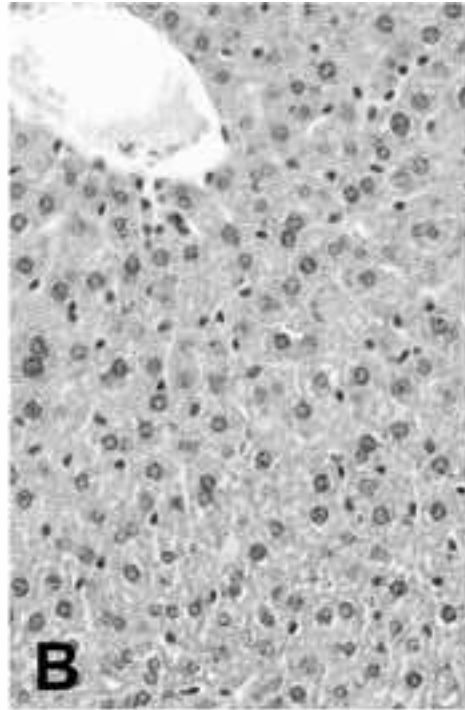
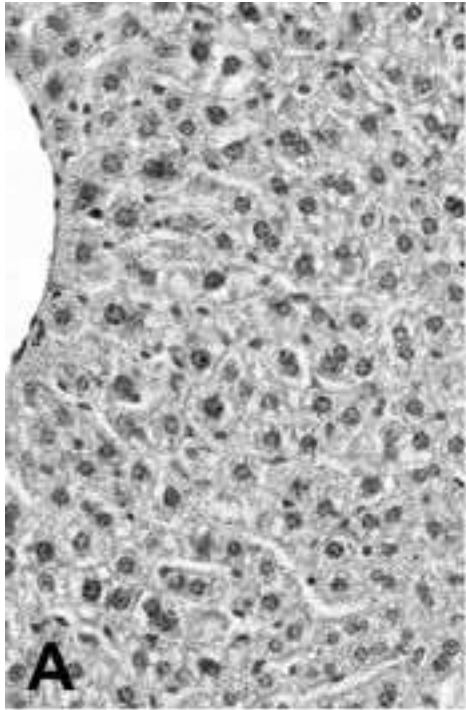


Fig. 2

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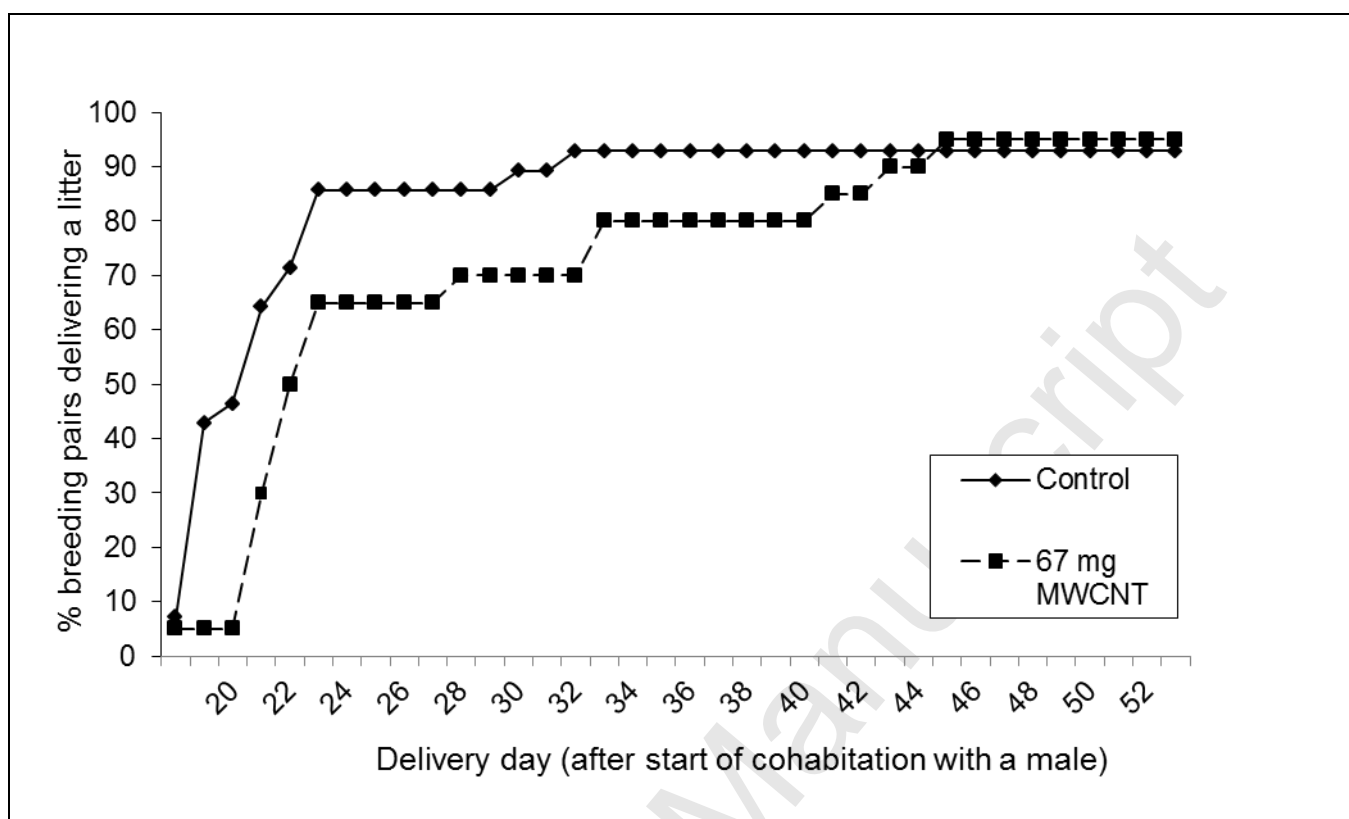


Fig. 5