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Oral two-generation reproduction toxicity study with NM-200 synthetic amorphous silica in Wistar rats

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ABSTRACT

Synthetic amorphous silica (SAS) like NM-200 is used in a wide variety of technological applications and consumer products. Although SAS has been widely investigated the available reproductive toxicity studies are old and do not cover all requirements of current OECD Guidelines. As part of a CEFIC-LRI project, NM-200 was tested in a two-generation reproduction toxicity study according to OECD guideline 416. Male and female rats were treated by oral gavage with NM-200 at dose levels of 0, 100, 300 and 1000 mg/kg bw/day for two generations. Body weight and food consumption were measured throughout the study. Reproductive and developmental parameters were measured and at sacrifice (reproductive) organs and tissues were sampled for histopathological analysis. Oral administration of NM-200 up to 1000 mg/kg bw/day had no adverse effects on the reproductive performance of rats or on the growth and development of the offspring into adulthood for two consecutive generations. The NOAEL was 1000 mg/kg body weight per day.

1. Introduction

Synthetic amorphous silica (SAS), a nanostructured material, is a form of silicon dioxide (SiO_2) which is produced for decades without significant changes in its physical–chemical properties. SAS is applied in a wide variety of technological applications and consumer products [1–4].

"Synthetic Amorphous Silica (SAS)" covers pyrogenic silica, silica gel and colloidal silica (EINECS No. 231-545-4).

The CEFIC sector group Association of Synthetic Amorphous Silica Producers recently summarized the properties of SAS [5]: 'SAS is produced by thermal (pyrogenic/fumed) or wet (precipitated, gel, colloidal) processes. In the initial particle formation step, primary particles with dimensions below 100 nm are formed by nucleation, coagulation and coalescence. These primary particles covalently bond to form indivisible units, called aggregates, which have no physical boundaries among them. The aggregates have external dimensions typically above 100 nm (pyrogenic, precipitated, gel). The aggregates combine to form agglomerates in the micron size range by physical attraction forces (van der Waals) and H-bridges. SAS powder is placed on the market as micron-sized agglomerates with an internal structure in the nanoscale. This fact is true for all currently known SAS products in powder form, independent of manufacturer, process and trade name. Colloidal silica is placed on the market as aqueous preparations of mono- and poly-dispersed nanoparticles.'

A recent investigation of Dekkers et al. [3] revealed that the total average daily intake of consumers via food is approximately 9.4 mg/kg bw/day, with 1.8 mg/kg bw/day being in the nano-size range.

Only limited information is available on the general toxicity of nanosilica after oral administration. Recently, the available studies have been reviewed by Dekkers et al. [3,6].

In a study of So et al. [7], Balb/c and C57BL/6 mice were fed diets containing 1% silica particles of 0.5–30 or 30–90 nm in size for 10 weeks. Although the daily oral dose was as high as 2 g/kg bw/day, only slight effects on alanine aminotransferase values were observed in the liver of Balb/C mice and some indications of a fatty liver were observed in both strains of mice.

In a more recent study of Van der Zande et al. [8], rats were given synthetic amorphous silica at dose levels of 100 or 1000 mg/kg body weight per day for 28 days or 2500 mg/kg body weight per day for

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84 days. The actual concentration of SAS in the nano-size range (5–200 nm) was 33, 328 and 819 mg/kg body weight/day, respectively. No effects on clinical signs and body weights were observed. Biochemical and immunological markers in blood and isolated cells did not indicate signs of toxicity. In the animals treated with the high dose of synthetic amorphous silica for 84 days, histopathologically, the incidence of periportal fibrosis was slightly, but not statistically significantly, increased.

In another recent oral 28 days toxicity study with nanosized silica, performed according to OECD Guideline 407, no adverse effects were observed after dietary administration of 1000 mg/kg body weight per day [9].

This is in agreement with the results of a 90 days toxicity study with colloidal silica nanoparticles recently reported by Kim et al. [10]. In this study, no treatment-related effects were observed in rats which were dosed by gavage with 500, 1000 and 2000 mg/kg body weight/day of nano silica of 20 and 100 nm.

Besides the effects of nanosized silica on general toxicity, an important endpoint under discussion in the context of nanotoxicity is potential reproductive effects of nanomaterials in parents and offspring. Evidence exists that (unborn) children may be more vulnerable than adults to exposure to chemicals as all organs are still in development and several experimental studies have shown that manufactured nanomaterials might adversely affect male and female reproduction and pre- and postnatal development (reviewed by Ema et al. [11]). SAS has been widely investigated in numerous toxicological studies including studies concerning reproductive toxicity [1,12]. There is no indication of reproductive toxic effects of orally dosed SAS based on these data.

Nevertheless, those studies were rather old and not performed according to regulatory requirements (OECD, EU, US-EPA guidelines). To fill this gap, as part of a CEFIC-LRI N3 project, an oral prenatal toxicity study according to OECD guideline 414 (which is reported separately by Hofmann et al. [13]) and a two-generation reproduction toxicity study according to OECD guideline 416 was performed with NM-200, a precipitated synthetic amorphous silica. The aim of the present study was to perform a two-generation reproduction toxicity study according to current OECD guideline 416 [14] and to examine the possible effects of SAS on the reproductive performance of rats and on the growth and development of the offspring into adulthood for two consecutive generations. For these studies the oral route was chosen because it is a major route of human exposure.

2. Materials and methods

2.1. General

This study was performed as part of a European Chemical Industry Council Long-range Research Initiative project (CEFIC-LRI N3 project) entitled 'Testing and Assessment of Reproductive Toxicity of Nanomaterials'.

The study was performed following the principles of the OECD Guideline for Testing of Chemicals 416 [14] and was conducted in accordance with the principles of Good Laboratory Practice (GLP) [15].

Animal care and use was in accordance with Directive 86/609/EEC, which establishes the general principles governing the use of animals in experiments of the European Communities, and with Dutch-specific legislation [16].

2.2. Test compound

NM-200 Synthetic Amorphous Silica (batch PR-A-2) was supplied by the Joint Research Centre (Ispra, Italy) and had the following characteristics: EINECS No. 231-545-4, CAS numbers 7631-86-9 (old general CAS number for silica including synthetic amorphous silica) and 112945-00-8 (CAS number for precipitated synthetic amorphous silica) and the purity was 96.5% (silicon dioxide content as SiO_2). NM-200 was stored at ambient temperature in the dark under N_2 atmosphere.

The vehicle, methylhydroxypropylcellulose (MHCP) (batch XH171907F1) was supplied by Dow Chemicals and had the following characteristics: CAS number 900-65-3 and the purity was 100% and MHCP was stored at ambient temperature. MHCP was also used as vehicle for NM-200 in general toxicity studies performed by Fraunhofer Institute of Toxicology and Experimental Medicine as part of the CEFIC-LRI N1 project (Tiered Approach to Testing and Assessment of Nanomaterial Safety to Human Health) (personal communication).

2.3. Animals

A total of 116 male and 116 female rats, Wistar outbred (Crl:WI(Han) strain) of about 4–5 weeks of age were obtained from a colony maintained under SPF conditions at Charles River Deutschland (Sulzfeld, Germany).

The animal room was kept under standard laboratory conditions with approximately 10 air changes per hour, temperature and the relative humidity controlled with target ranges of $22 \pm 2\,^{\circ}C$ and 45–65%, respectively and a 12-h light cycle.

A rodent breeding diet (Rat and Mouse 3, Special Diets Services, Witham, England) and water from the public supply were available *ad libitum*. The diet in the feeders was refreshed once per week and topped up when necessary. Animals were housed in macrolon cages with a bedding of wood shavings (Lignocel, Rettenmaier, Rosenberg, Germany) and strips of paper as environmental enrichment (Enviro-dri, Lillico, Betchworth, England).

2.4. Test formulations

Once weekly, until completion of the dosing period of the study, seven bottles per dosing group were prepared, each containing the appropriate amount of NM-200 and stored at ambient temperature in the dark under N_2. On each subsequent day, the required amount of vehicle (0.5% v/v of MHPC in Ultrapure water) was added to achieve concentrations of 0, 10, 30 and 100 mg/ml NM-200 and stirred on a magnetic stirrer (approximately 900 rpm) for at least 60 min. All samples were continuously stirred under the same conditions during the entire daily administration period in order to maintain the homogeneity of the NM-200 in the vehicle.

At various weeks during the study samples were taken from each of the dosing formulations for analytical investigations of the hydrodynamic diameter of the SiO_2 particles using Dynamic Light Scattering (DLS) technique.

2.5. Analysis of NM-200

The particle size and the particle size distribution of the NM-200 particles in the vehicle MHPC were analyzed with a Zetasizer-Nano ZS Instrument (Malvern). Dynamic Light Scattering (DLS) used to determine the size distribution of the particles is based on the quantification of dynamic fluctuations of light scattering intensity caused by Brownian motion of the particles. This technique yields a hydrodynamic diameter that is calculated via the Stokes–Einstein equation from the aforementioned measurements. The result of the measurements is the average hydrodynamic diameter of the particles, the peak value is the hydrodynamic diameter distribution and the polydispersity index (PDI) that describes the width of the particle size distribution. The PDI scale ranges from 0 to 1, with 0 being monodisperse and 1 being polydisperse. Each assigned size

and PDI was the mean of 9 sub runs. All measurements were carried out in triplicate with a temperature equilibration time of 1 min at 20 °C.

2.6. Dosing of animals

The test item NM-200 was administered to the animals by oral gavage at concentrations of 0, 100, 300 and 1000 mg/kg body weight/day. Male animals were dosed during a 10-week premating period and during mating and up to the day before sacrifice. Female animals were dosed during a 10-week premating period and during mating, gestation and lactation up to postnatal day 21. Selected F1-generation pups were dosed by gavage from postnatal day 22 until the day prior to sacrifice. The dose volume was 10 ml/kg body weight. Dose volumes were adjusted to the latest recorded body weight for each individual animal to maintain a constant dose level in terms of the animal's body weight. During the gestation period, dose volume was not adjusted after gestation day 14.

2.7. Study design

Three days before the start of the study, all male and female rats were weighed and allocated to four groups of 28 rats/sex/group by computer randomization on the basis of mean body weight and housed four sex/group. All rats were dosed by gavage from the start of the 10-weeks premating period. At the end of this period the males were housed individually and a female was placed with a male of the same dosing group, according to numerical sequence (F0-generation). Every morning (during a 2 weeks mating period) the females were checked for evidence of mating. The day of detection of sperm cells in the vaginal smear was considered day 0 of gestation and the mated F0 females were housed individually during a 3 weeks gestation period. During the lactation period the females and their litters (F1-generation) remained in the same cages.

The morning after birth was considered postnatal day 1 (PN day 1). Consequently, for litters that were born during the day, but after the morning observation, that day was considered PN day 0.

On PN day 4, litters of more than eight pups were adjusted by eliminating (culling) extra pups by random selection to yield, as nearly as possible, four males and four females per litter.

On PN day 21, the F1 pups were weaned and shortly thereafter 28 males and 28 females were selected at random from as many litters as possible in each group to produce the next generation. Mating of siblings was avoided. Of the remaining F1 pups, one male and one female pup of each litter was subjected to a thorough necropsy (see below). The F1 animals selected to produce the F2 generation were housed four sex/group and treated at the same dose levels as their parents from the day of weaning until sacrifice. These animals were mated at the end of the premating period of at least 10 weeks. The procedure followed to rear the F2 generation litters were identical to those described for the F0 generation to rear the F1 generation.

In the selected F1-animals, sexual maturation was studied by scoring the day of vaginal opening in females and testes descent and preputial separation in males from PN days 31, 21 and 39 postpartum onwards, respectively.

Three weeks prior to the end of the premating period of the F0- and F1 generation, vaginal smears were made of each female to evaluate the oestrous cycle length and normality.

2.8. Observations

Throughout the study, all animals were checked daily for clinical signs and abnormal behavior.

The body weight of all males and females was recorded weekly during the premating period and the body weight of males weekly thereafter until sacrifice. Mated females were weighed on gestation days 0, 4, 7, 10, 14, 17 and 21 and during lactation on PN days 1, 4, 7, 10, 14, 17 and 21. In addition, the animals were weighed on their scheduled necropsy date in order to calculate the correct organ to body weight ratio.

Throughout the premating period, food consumption was measured weekly for each cage by weighing the feeders. Individual food consumption of all mated females were recorded from gestation days 0–4, 4–7, 7–10, 10–14, 14–17 and 17–21 and for females with live pups from postpartum days 1–4, 4–7, 7–10, 10–14, 14–17 and 17–21.

Water consumption was not measured.

For assessment of the fertility and reproductive performance, the mating, fertility, fecundity, gestation, live births, viability and lactation indices were calculated and the sex ratio of pups was determined (for definitions of indices, see footnotes to Tables 2 and 3). For each litter, the litter size (dead and live pups), number of male and female pups, and the number of pups with external abnormalities were determined on PN days 1, 4, 7, 10, 14, 17 and 21. The pups were weighed individually on PN days 1, 4, 7, 10, 14, 17 and 21. A necropsy was performed on stillborn pups and on pups that died during lactation.

2.9. Pathological examinations

At or shortly after weaning one male and one female F0 and F1 pup per litter were subjected to a thorough necropsy. Hereto, pups were euthanized by exsanguination from the abdominal aorta under CO_2/O_2 anesthesia and then examined grossly for pathological changes and the brain, spleen and thymus were weighed.

At termination, all surviving parental animals of the F0 and F1 generation were euthanized by exsanguination from the abdominal aorta under CO_2/O_2 anaesthesia and then examined grossly for pathological changes. The male animals were subjected to sperm analysis. Hereto, caudal epididymal sperm count and sperm motility and testicular sperm count were measured using the Hamilton Thorne Integrated Visual Optical System (IVOS). In addition, epididymal sperm morphology was examined. The adrenals, brain, epididymides, kidneys, liver, ovaries, pituitary gland, prostate, seminal vesicles with coagulating glands, spleen, testes, thyroid, uterus with cervix (after counting of implantation sites), vagina and all gross lesions were preserved for microscopical examination and all were weighed except for the vagina. All organs were preserved in a neutral aqueous phosphate-buffered 4% solution of formaldehyde except for the testis which were preserved in Bouin's fixative.

The tissues and organs were prepared for microscopic examination using standard procedures and stained with haematoxylin and eosin, except for the sections of the testes which were stained with PAS haematoxylin. Microscopic examination was carried out on the organs of all rats of the control (0 mg/kg bw) and high-dose group (1000 mg/kg bw) and on the macroscopic abnormalities of all groups. In addition, the reproductive organs of the F0 and F1 males that failed to sire and of the non-mated and non-pregnant females of the low- (100 mg/kg bw) and mid-dose (300 mg/kg bw) group were examined.

2.10. Statistical analyses

The statistical keys used to analyze all the parameters measured in this study are described in the legends of the Tables showing the summarizing data.

Table 1
Results of the size and size distribution analysis of the 0, 10, 30 and 100 mg/ml NM-200 formulations sampled at different time points during the study.

Sample	Concentration (mg/ml)	Average size (nm)	PDI
Start mating period F0-generation	0*	–	–
	10	1076	0.93
	30	912	0.68
	100	695	0.75
Premating period F1-generation	0*	–	–
	10	1247	0.75
	30	1032	0.72
	100	703	0.36
Start mating period F1-generation	0*	–	–
	10	1475	1.00
	30	1216	0.85
	100	522	0.67
Last week of the study	0*	–	–
	10	1664	1.00
	30	876	0.54
	100	409	0.95

* Control samples showed multiple peaks with low intensity which does not interfere with particle measurement.

3. Results

3.1. Analysis of NM-200 particles

The mean hydrodynamic diameter of the SiO_2 particles in the 10 mg/ml study samples varied between 1076 and 1664 nm and for the 30 mg/ml study samples between 876 and 1216 nm, respectively. The measured size of the 100 mg/ml study samples appeared to be the smallest (409–703 nm) but due to the high concentration of the particles in the samples they were sedimentated and aggregated. Furthermore, multiple scattering occurred in these samples which also influenced the measurements (Table 1).

3.2. Clinical signs and mortality

No treatment-related mortality was observed in the animals of the F0 and F1 generation orally exposed with NM-200. Furthermore, observations of the F0 and F1 generation animals during the premating, gestation and lactation periods did not show any differences in the animals' appearance, general condition or behavior among the treatment and control group (data not shown).

3.3. Body weight and body weight change

No treatment related effects were observed on the body weights and body weight changes of the male animals of the F0 and F1 generation and the female animals during the premating, gestation or lactation period of the F0 and F1 generation among the control and treatment groups (data not shown).

3.4. Food consumption

No treatment related effects were observed on the food consumption of the male animals of the F0 and F1 generation and the female animals during the premating, gestation or lactation period of the F0 and F1 generation among the control and treatment groups (data not shown).

3.5. Fertility and reproductive performance

Fertility and reproductive performances are presented in Table 2. Except for one female of the control group of the F0 generation, all females of all groups for both generations were mated, the mating index ranged from 96 to 100%. Pre-coital time and gestation times were similar among all groups for both generations. The female fertility and fecundity indices ranged from 96 to 100% and 89–98% for the F0 and F1 generation, respectively. The gestation index ranged from 96 to 100% for both generations. One female of the low-dose group of the F0 generation delivered only dead pups. Post-implantation loss ranged from 8.6 to 10.5 and 8.9–17.7 for the F0 and F1 generation, respectively.

3.6. Offspring data

Offspring data of the F0 and F1 generation are presented in Table 3. The total number of pups delivered was similar among the groups for both generations. No effects were observed on live birth index, pup mortality on day 1 and on the viability indices on day 4 and 21 for both generations. The number of litters that were lost entirely accounted 2, 2, 2, 0 and 1, 3, 1, 4 for the control, low-, mid- and high-dose groups of the F0 and F1 generation, respectively. The sex ratio for both generations was within the normal range for this laboratory. For both generations no effects of NM-200 were observed on pup body weights and on the absolute and relative weight of the brain, thymus and spleen of male and female pups (data not shown).

3.7. Sexual maturation

Results of testes descending and preputial separation in male animals of the F1 generation and of vaginal opening of the female animals of the F1 generation are shown in Table 4. No differences in the sexual maturation were found in the F1 animals among the NM-200 groups and the control group.

3.8. Oestrous cycle length

No differences in oestrous cycle length or normality of the oestrous cycle were observed between the female animals of the control group and the NM-200 groups for the F0 and F1 generation (Table 5).

3.9. Epididymal and testicular sperm

No differences in epididymal sperm motility, epididymal sperm count, epididymal sperm morphology, testicular sperm count and daily sperm production were observed between the male animals of the control group and the NM-200 groups for the F0 and F1 generation (Table 6).

3.10. Necropsy

Except for a statistically significant decreased relative weight of the thyroid of the male animals of the mid-dose group of the F1-generation, no statistically significant differences were observed between absolute (data not shown) and relative organ weights of male and female animals of the various groups of the F0- and F1-generation (Table 7).

At necropsy no treatment-related macroscopic changes were observed in the selected weanlings and parental animals of the F0 and F1 generation (data not shown). Microscopical examination did also not reveal any treatment-related histopathological changes

Table 2
Reproductive performance of female rats receiving NM-200.

Parameter of reproductive performance	Generation	NM-200 (mg/kg body weight/day)			
		0	100	300	1000
Mating index (%)[a]	F0	96	100	100	100
	F1	100	100	100	100
Fertility index (%)[b]	F0	96	100	96	96
	F1	98	93	93	89
Fecundity index (%)[c]	F0	100	100	96	96
	F1	89	93	93	89
Gestation index (%)[d]	F0	100	96	100	100
	F1	100	100	96	96
Precoital time (days)[e]	F0	2.7 ± 0.21	2.8 ± 0.25	2.3 ± 0.19	2.5 ± 0.21
	F1	2.8 ± 0.27	2.5 ± 0.20	2.4 ± 0.20	2.4 ± 0.26
Gestation time (days)[e]	F0	21.7 ± 0.09	21.6 ± 0.11	21.6 ± 0.10	21.6 ± 0.10
	F1	21.3 ± 0.09	21.2 ± 0.08	21.4 ± 0.12	21.3 ± 0.09
Postimplantation loss per animal (%)[e,f]	F0	8.6 ± 2.66	10.5 ± 3.72	9.5 ± 2.54	10.1 ± 2.52
	F1	8.9 ± 1.48	13.6 ± 3.68	17.6 ± 5.47	14.8 ± 4.91

[a] (No. of females mated/no. of females placed with males) × 100.
[b] (No. of females pregnant/no. of females placed with males) × 100.
[c] (No. of females pregnant/no. of females mated) × 100.
[d] (No. of females with live pups/no. of females pregnant) × 100.
[e] Values are means ± SEM; statistical test: Kruskal–Wallis + Mann–Whitney U test.
[f] Total postimplantation loss = ((No. of implantation sites − no. of pups born alive)/no. of implantation sites) × 100.

Table 3
Offspring data from rats receiving NM-200.

	Gestation	NM-200 (mg/kg body weight/day)			
		0	100	300	1000
Pups delivered (total)[a]	F0	10.3 ± 2.9	10.7 ± 2.3	11.3 ± 2.0	11.0 ± 1.5
	F1	11.5 ± 1.6	10.7 ± 2.8	10.4 ± 2.8	11.0 ± 2.6
Live birth index (%)[a,c]	F0	97.3 ± 9.9	94.8 ± 19.1	96.5 ± 12.7	98.7 ± 4.9
	F1	96.2 ± 6.1	90.7 ± 17.3	94.2 ± 12.7	93.5 ± 16.1
Pup mortality day 1 (%)[a]	F0	2.7 ± 9.9	5.2 ± 19.1	3.5 ± 12.7	1.3 ± 4.9
	F1	3.8 ± 6.1	9.3 ± 17.3	5.8 ± 19.0	6.5 ± 16.1
Viability index day 4 (%)[a,d]	F0	99.3 ± 2.5	99.6 ± 2.0	98.9 ± 4.2	98.8 ± 3.5
	F1	84.7 ± 28.0	83.8 ± 34.6	95.3 ± 20.0	73.8 ± 42.3
Viability index day 21 (%)[a,e]	F0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
	F1	100 ± 0	99.5 ± 2.6	98.6 ± 6.8	100 ± 0
Whole litter losses[b] (litters lost/total number of litters)	F0	2/27	2/28	2/27	0/27
	F1	1/25	3/26	1/25	4/24
Sex ratio day 1 (%)[f]	F0	51.9 ± 17.6	43.9 ± 12.2	50.8 ± 15.6	51.1 ± 10.9
	F1	47.4 ± 16.2	52.6 ± 21.4	45.3 ± 19.6	42.3 ± 15.4

[a] Values are means ± SD per litter. Statistical test: Kruskal–Wallis followed by Dunnett's multiple comparison test.
[b] Statistical test: Fisher exact test.
[c] (No. of pups born alive/no. of pups born) × 100.
[d] (No. of pups at day 4/no. of pups at day 1) × 100.
[e] (No. of pups at day 21/no. of pups at day 4) × 100.
[f] % of male pups of total live pups at day 1. Statistical test: Anova followed by Dunnett's multiple comparison test.

Table 4
Effect of NM-200 on sexual maturation in F1-generation pups.

	Number of animals evaluated	NM-200 (mg/kg body weight/day)			
		0	100	300	1000
		Mean age of reaching criteria (PN days) (mean ± SEM)			
Testes descending	28	26.3 ± 0.3[a]	26.4 ± 0.3	25.8 ± 0.3[a]	25.9 ± 0.4
Preputial separation	28	43.5 ± 0.3	44.3 ± 0.7	44.3 ± 0.6	45.3 ± 0.8
Vaginal opening	28	35.2 ± 0.6	35.4 ± 0.9	35.2 ± 0.5	35.6 ± 0.5

Statistics: ANOVA followed by Dunnett's multiple comparison test.
[a] Testes of one male pup of the control group and one male of the 300 mg/kg bw/day group were not descended before PN day 43. After this day, the animals were not checked anymore but both animals were able to sire.

Table 5
Effect of NM-200 on oestrous cycle length of rats.

Oestrous cycle length (days)	Generation	NM-200 (mg/kg body weight/day)	
		0	1000
		Number of females	
	F0	28	28
	F1	28	28
4	F0	27	27
	F1	25	25
5	F0	1	1
	F1	2	1
>5	F0	0	0
	F1	0	1

Statistics: chi-square test.
Since no effects on oestrous cycle length was observed in the high dose group oestrous cycle analysis was not performed in the intermediate groups.

in the selected weanlings and parental animals of the F0 and F1 generation (data not shown).

4. Discussion

As part of a CEFIC-LRI project 'Testing and Assessment of Reproductive Toxicity of Nanomaterials' a two-generation reproduction toxicity study (OECD technical guideline 416 [14]) with NM-200 precipitated synthetic amorphous silica was performed. A prenatal developmental toxicity study (Hofmann et al. [13]) with the same test compound which was also conducted as part of this project is reported separately.

In the present two-generation reproductive toxicity study, the test compound synthetic amorphous silica NM-200 was administered to Wistar rats by gavage at concentrations of 0, 100, 300 and 1000 mg/kg body weight/day to evaluate its potential on the reproductive performance of rats or on the growth and development of the offspring into adulthood for two consecutive generations.

In this study, the test-item was dosed as delivered in an agglomerated aggregated form. The agglomerates were broken down in dispersion using MHPC by stirring for at least 1 h the dosing samples. The resulting mean hydrodynamic diameter of NM-200 particles ranged between 1076 and 1664 nm for the low-dose group (10 mg/ml) and 876–1216 nm for the mid-dose group (30 mg/ml) while the particles in the dispersions of the highest concentration of 100 mg/ml sedimented and agglomerated. These sizes are consistent with previous results showing that, in general, precipitated synthetic amorphous silica consists of agglomerates of aggregates typically much larger than 100 nm (>1 μm). Aggregates or agglomerates smaller than 100 nm do not generally liberate from these agglomerates [4]. Besides, as described before, consumers are mainly exposed to silica particles with a size greater than the nanosize; 80% of the average daily intake of silica by consumers via the food is greater than 200 nm [3].

In other, mainly *in vitro*, studies thorough sonification of the dispersions with 10% serum in medium as a vehicle resulted in particle dispersions with significant smaller mean diameters. Also in the prenatal toxicity study which was performed as part of this project much smaller mean diameters of the NM-200 particles in the dispersions were reported. In that study a vehicle containing 10% fetal calf serum was used (Hofmann et al. [13]).

Also a report of the Joint Research Centre on the characterization of the NM-200 reference material shows a smaller size distribution of NM-200 agglomerates in Modified Eagle medium using a relative high concentration of serum proteins [17].

In the present study MHPC was used as a vehicle instead of fetal calf serum (FCS) because the possible effects of long-term treatment of animals with 10% FCS are not known but prolonged oral treatment with a high concentration of FCS might be detrimental for animals. The use of a vehicle containing 10% FCS in the present study implied that the animals would be exposed, for a prolonged period, to a relative high concentration of (undefined) proteins that could affect, among others, the nutritional status of the animals and/or could result in harmful effects on the kidneys. Besides, FCS contains a high concentration of various growth factors that, after prolonged administration, might also influence normal physiology of the animals. When using an uncommon vehicle as FCS, with a high concentration of proteins and growth-factors, a second control group should be used (without FCS) to check for these, and other, possible adverse effects of FCS. For those reasons, for this study, it was decided to use a more common cellulose-based vehicle that is frequently used in this kind of toxicity studies in case the test item is likely to precipitate.

The optimal vehicle for separation of the particles to achieve smallest particles sizes may be different than the optimal vehicle suitable for long-term (toxicity) studies with animals. The

Table 6
Effect of NM-200 on sperm parameters.

Sperm parameter	Generation	NM-200 (mg/kg body weight/day)			
		0	100	300	1000
Epididymal sperm motility (%)[a]	F0	74.0 ± 2.2	75.7 ± 1.5	74.9 ± 1.6	76.3 ± 1.3
	F1	78.5 ± 2.4	82.0 ± 1.5	78.8 ± 1.5	80.3 ± 1.4
Epidydymal sperm count (10^6/g)[b]	F0	104.4 ± 11.6	92.3 ± 6.5	92.5 ± 6.6	108.3 ± 10.5
	F1	68.9 ± 6.8	78.4 ± 6.1	93.3 ± 9.8	88.1 ± 9.7
Testicular sperm count (10^6/g)[b]	F0	78.8 ± 3.8	nm	nm	82.5 ± 3.9
	F1	69.2 ± 5.7	nm	nm	72.0 ± 4.8
Daily sperm production (10^6/day)[b,c]	F0	12.9 ± 0.6	nm	nm	13.5 ± 0.6
	F1	11.3 ± 0.9	nm	nm	11.8 ± 0.8
Sperm morphology[a]					
Normal (%)	F0	99.80 ± 0.35	nm	nm	99.64 ± 0.38
	F1	99.13 ± 1.10	nm	nm	98.98 ± 0.94
Affected (%)	F0	0.20 ± 0.35	nm	nm	0.36 ± 0.38
	F1	0.88 ± 1.10	nm	nm	1.02 ± 0.94

[a] Statistics: Kruskal–Wallis non-parametric analysis of variance followed Mann–Whitney U test.
[b] Statistics: ANOVA followed by Dunnett's multiple comparison test.
[c] (No. of spermatozoa per gram testicular parenchyma/6.1) nm: since no effects on these parameters were observed in the high dose group they were not analyzed in the intermediate groups.

Table 7
Effect of NM-200 on relative organ weights of F0 and F1 parental animals.

Organ	Generation	NM-200 (mg/kg bw/day)							
		0 (control)		100		300		1000	
		Male	Female	Male	Female	Male	Female	Male	Female
		Mean organ weight relative to terminal body weight (g/kg body weight)							
Adrenals	F0	0.163	0.311	0.163	0.313	0.160	0.312	0.164	0.294
	F1	0.166	0.316	0.161	0.304	0.165	0.308	0.164	0.308
Brain	F0	5.041	6.818	5.156	6.917	5.152	6.838	5.146	6.761
	F1	5.311	6.775	5.221	6.909	5.305	6.850	5.247	6.815
Epididymides	F0	2.959	–	3.083	–	3.187	–	3.116	–
	F1	3.327	–	3.305	–	3.247	–	3.237	–
Kidneys	F0	5.817	6.715	5.927	6.720	5.834	6.640	5.941	6.532
	F1	5.823	6.469	5.846	6.266	5.738	6.275	5.729	6.287
Liver	F0	29.601	45.689	28.835	45.519	29.474	45.953	29.375	47.091
	F1	29.899	44.364	29.622	43.932	29.933	43.945	29.630	43.793
Ovaries	F0	–	0.346	–	0.342	–	0.357	–	0.313
	F1	–	0.341	–	0.363	–	0.330	–	0.347
Pituitary gland	F0	0.039	0.058	0.040	0.055	0.040	0.055	0.037	0.058
	F1	0.033	0.046	0.033	0.049	0.032	0.047	0.033	0.050
Prostate	F0	2.470	–	2.389	–	2.504	–	2.410	–
	F1	2.530	–	2.577	–	2.405	–	2.262	–
Seminal vesicles	F0	3.270	–	3.208	–	3.179	–	3.199	–
	F1	3.043	–	3.247	–	3.154	–	3.145	–
Spleen	F0	1.835	2.284	1.618	2.209	1.652	2.286	1.661	2.148
	F1	1.775	2.168	1.801	2.112	1.833	2.131	1.704	2.147
Testes	F0	8.567	–	9.018	–	8.968	–	8.901	–
	F1	9.411	–	9.200	–	9.371	–	9.167	–
Thyroid	F0	0.062	0.074	0.064	0.074	0.063	0.072	0.064	0.071
	F1	0.061	0.069	0.055	0.069	0.053*	0.068	0.056	0.067
Uterus	F0	–	1.914	–	2.231	–	2.206	–	1.983
	F1	–	1.992	–	2.091	–	2.007	–	1.920

Statistics: ANOVA followed by followed by Dunnet's multiple comparison test.
* $P < 0.05$.

analysis of size distribution of the particles used is essential for toxicity studies with nanomaterials but will take more resources than for analysis of traditional industrial chemicals.

Generally, in this study no toxicological-relevant differences were observed between rats receiving 100, 300 or 1000 mg/kg body weight/day of synthetic amorphous silica and those receiving the vehicle only. No test item related effects were observed on clinical signs, mortalities, body weight and food consumption. Furthermore, no effects were observed on fertility and reproductive parameters including the mating, fertility, fecundity and gestation indices, pre-coital and gestation times, pre- and post-implantation losses and sex ratios. No oestrous cycle irregularities were observed and all sperm parameters measured were similar among the various groups. No treatment related effects were observed on any of the development parameters including pup viability indices, pup weights, pup organ weights and the sexual maturation measurements on testes descending, preputial separation and vaginal opening.

At sacrifice of the F0- and F1-generation animals, no toxicological relevant differences were observed on absolute- and relative organ weights and on macroscopical and microscopical examinations.

In conclusion, under the conditions of this study, administration of synthetic amorphous silica NM-200 during two generations at concentrations up to 1000 mg/kg body weight/day had no effects on reproduction of the parental F0 and F1 generations animals nor on the development of the F1 and F2 pups, nor on the sexual maturation of the F1 weanlings. Based on the results of the present study The No Observed Adverse Effect Level (NOAEL) was established at 1000 mg/kg body weight per day.

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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