

Letter Regarding “Chronic Oral Exposure to Synthetic Amorphous Silica (NM-200) Results in Renal and Liver Lesions in Mice”



To the Editor: Boudard *et al.*¹ in “Chronic Oral Exposure to Synthetic Amorphous Silica (NM-200) Results in Renal and Liver Lesions in Mice” described lesions in the kidneys and livers of mice exposed to NM-200, a synthetic precipitated amorphous silica (SAS). The lesions occurred at a low-dose exposure taken up by drinking water. Three experiments are described in this publication, which together tend to highlight a specific behavior of the tested substance; however, having some scientific deficiencies that jeopardize the overall drawn conclusion. The authors have performed 3 experiments in which small groups of animals were exposed via drinking water starting at an age of 3 months over a long period up to 18 months. Three experiments have been performed according to the described study design. The study design has been described as “[The] experiment was carried out in parallel using 2 different wild-type mouse lines, namely C57BL/6 and C57BL/6S. Besides, a 9-month exposure study was performed in a transgenic mouse line (TgHuA53T) expressing the human mutated (A53T) α -synuclein protein.”¹ The identified scientific and study design deficiencies of the study are described in this letter to the editor.

STUDY DESIGN

Selection of Strains

Interestingly, the authors mentioned that the mouse lines C57BL/6 (Charles River, France) and C57BL/6S (Harlan, France), were “selected on the criteria of absence of any spontaneous diseases, in particular no kidney or liver tumors that could appear with natural aging.”¹ This statement is insofar surprising because especially in males, liver tumors are not unusual at all.^{2,3,S1-S3} Liver neoplasms were even considered the second most common tumors in C57BL/6 mice.^{S1} Furthermore, the animals were placed into this study at an age of 3 months and were maintained for a further 18 months (i.e., the animals are senile individuals at the end of the study).

In addition, the statement that C57BL/6 mice do not express spontaneous renal diseases is wrong. Nephropathy was among the most common non-neoplastic findings that contributed to death in long-term survival studies of *ad libitum* fed and restricted diet feeding in C57BL/6 mice.² The most common non-neoplastic findings in another study included glomerulonephritis.^{S3} Furthermore, the genetic background of C57BL/6 mice was used to establish the renal phenotype of the cystinosis mouse model.^{S4}

General Study Design

Regarding the study design, it is stated that groups of “5 to 8 female mice, 3 months old at the beginning of the experiment (average weight 20 to 25 g), were exposed orally to NPs through their drinking water for 18 months” and “[c]ontrol group of each mouse line ($n = 7$ and $n = 8$) received only tap water during the 18 months.”¹ In addition, a third experiment was performed “to study the impact of 3-, 6-, and 9-month exposure to silica in drinking water on young (8 weeks old, average weight 20 to 25 g) transgenic mice ($n = 15$, male and female)” expressing the human mutated (A53T) α -synuclein protein (TgHuA53T) “compared [to] the matched controls ($n = 10$, unexposed transgenic mice).”¹ No information has been provided on the silica content in the tap water and diet and thus about the background magnitude already present through normal water and diet consumption (see the section “Silica Content in Different Organs,” later in this article). It is also well known that rodent feed for mice and rats is enriched with certain vitamins that usually use synthetic amorphous silica as a carrier material and thus create a second background, again no data were provided on this aspect neither on the amorphous silica concentration in the used feed nor about the feed consumption of each animal on average.

Even, for a special study design, where no standard guidance is used as a basis and besides of performing such an impacting study outside GLP, it is unusual that mice were used that were 3 months of age at study start. It surprises even more that a such low number of animals have been used in such a long experiment, knowing that a huge number of age-related background lesions, and even mortality, will interfere with the study results.

It is furthermore unclear how the daily SAS consumption was evaluated considering a water uptake of “average daily water intake is estimated to be around 3–5 mL (1.5 mL/10 g body weight/day)”¹ The authors declared furthermore that “the usual estimated

Table 1. Summary on study design

Strain	Exposure phase (Supplementary Figure S1)	Liver histopathology table (Supplementary Table S3)	Histo summary table (Table 1)
C57BL6-C	7	<i>n</i> = 2 age matched, <i>n</i> = 7 young adult	<i>n</i> = 7 old and <i>n</i> = 9 young adult
C57BL6/S-C	8	8	<i>n</i> = 6 old
C57BL6-NP	8	6	3
C57BL6/S-NP	5	5	5

individual intake of 4 mL/day was used to calculate the average daily intake of mice.¹ One might ask the question, was it measured or estimated. But if it was estimated, it is questionable to declare a daily SAS uptake of “4.8 mg SiO₂/kg body weight/day.”¹

PATHOLOGY EVALUATION

Missing Animal Data

Although no mortality was reported (except in a table for TgHuA53T mice [Supplementary Table S4], without evaluation of the cause of death) during the conduct of the study, it remains unclear why obviously several organs have not been evaluated regarding histopathological changes or Si tissue distribution. It was necessary to compile a table (see Table 1) for the C57BL6/S and C57BL6 mice to get an overview on the evaluation of animals. In Supplementary Figure S1 of the publication, the authors describe the experimental setup with animal numbers per group. In Supplementary Table S3 and Table 1, histopathological results are reported for part of exposed animals in C57BL6 and C57BL6/S nanoparticle exposed animals. Information on the selection of animals is missing. It further remains unclear how 2 age-matched animals were included in the experiment in C57BL6 Control only and how they were treated. It gets even more obscure as in the publication Table 1 this changes to 7 old and 9 young adult mice (Table 1).

It is unclear, what “age matched” does mean (Table 1). In the supplementary document, it is stated that TgHu53T mice were used to study the impact of 3, 6, and 9 months exposure on young mice at an age of 8 weeks. It may be considered that transgenic mice have entered the study at an age of 8 weeks and the sentence “compared with the matched controls (*n* = 10, unexposed transgenic mice)” is a statement of using controls at the same age. This is reasonable but is different from the main document statement on mice entering the study at an age of 3 months.

Evaluation

A 3-score system has been applied to describe the lesions: “Grade 1, for 0% to 25% of lesions observed in the section (+); Grade 2, for 25% to 50% of lesions

observed in the section (++)”; Grade 3, when there was more than 50% of lesions in the section (+++)”¹ Such scoring scheme is not considered adequate to differ induced lesions from spontaneous background changes nor to properly classify lesions. The STP Best Practice Paper on pathology report writing recommends: “When severity grading is important to the understanding of major study findings, it may be useful to provide a description of the distinguishing features of each severity grade.”⁵⁵ Furthermore, a 5-grade system (beside of 0, where no findings are present) has been recommended by several authors,⁵⁶ but also by the INHAND-Nomenclature.⁵⁷

Renal Changes

The authors stated that “No morphological abnormalities were noted on young adult or age-matched control mice.”¹ This sentence astonishes likely every, even little experienced toxicologic pathologist. Renal background alterations are common in every mouse strain, and, especially in aged animals; histological lesions are accumulating in any rodent species and strain. Moreover, changes in the urinary system in C57BL/6 mice are, for example, summarized as mouse urologic syndrome by Szymanska *et al.*⁵¹ Nephrotic lesions were also reported by Brayton *et al.*⁵⁸

Vacuolar Changes

The authors stated that the observation of findings in kidneys as “[v]acuoles within tubules, especially proximal tubules, were seen in all the animals exposed to NM-200 whatever the mouse line, without atrophic lesions or tubular necrosis (Figure 2h)”¹ and “unrelated to normal aging, that were characterized by a vacuolization of tubular epithelial cells, detected in 100% of the kidneys.”¹ Figure 2H, however, is described as “[r]epresentative of silver impregnation that illustrates absence of atrophic lesions or tubular necrosis.”¹ However, no vacuolization of tubular cells is visible. This is also true for any other picture presented under Figure 2. In contrast, a focal vacuolar change in the renal tubules is presented in Supplementary Figure S4C and D. Such lesions are, however, often noted changes in mouse kidneys and are considered an incidental finding,^{59,60} especially when not associated with necrosis or atrophy.

Glomerular Changes

Although the C57BL/6 mouse is relatively resistant toward developing glomerulosclerosis, proteinuria, and hypertension, it is known to represent a strain that is used to establish nontransgenic models for induced renal injury.^{61,62} In addition, very detailed evaluations have been undertaken to investigate glomerular changes in C57BL/6 mice during live time.^{63,64}

Yumura *et al.*^{S14} have shown age-related glomerular changes by demonstrating increased glomerular injury associated with a marked deposition of IgG and IgM in the enlarged mesangium from CB57BL/6 mice during ages of 6 to 24 months. In addition, Yabuki *et al.*^{S13} have undertaken a very detailed image analysis. They could show that the number of glomeruli per unit area does not change at ages 3 to 15 months, but thereafter, gradually increase in mice aged 24 to 27 months of age. They considered this numerical change as a possible consequence of tubular atrophy. Furthermore, they evaluated the age-related glomerular changes. Briefly, the percentage of glomeruli with a cuboidal parietal layer decreased, and the cuboidal layer of the renal capsule in males displayed atrophic changes during the later stages of life. In addition, glomerular lesions that were heavily or mildly stained with PAS (expanded mesangium) were rarely encountered in 3-month-old animals but became severe and diffuse with age. Other changes consisted of interstitial inflammatory cell infiltration by lymphocytes and plasma cells, amyloid deposition, scar lesions in the cortex, and ultrastructural changes that were observed in mice at 27 months old. Ultrastructural changes revealed in aged mice severe expansion of the mesangial matrix, fusion of podocyte foot processes, and accumulation of lysosomes and large lysosomes with accumulated lipid content.

Amyloidosis

The authors described amyloidosis in the renal glomeruli for 1 of 5 C57BL/6S mice and 1 of 3 C57BL/6 mice. In Figure 2c–e, hyaline deposits in glomeruli are shown. The Congo red stained negative (Figure 2b), and hence, hyaline glomerulopathy can be excluded.^{S15} There is therefore no doubt, that the cases (see also “liver”) present true amyloidosis. However, amyloidosis is a very common age-related background lesion in mice. In contrast to, for example, A/J mice, the C57BL/6 strains are some of the most susceptible mouse strains.^{S16,S17} Amyloidosis was reported also by other authors that investigated mouse kidneys in detail.^{S13,S18} Some others considered amyloidosis the most common age-related non-neoplastic finding with >80% of C57BL/6 males affected.^{S3}

The reported incidence is not deemed to be very high, considering, for example, published incidences by Zurcher *et al.*^{S3} of 73% in females and 83% in males. In general, C57BL6 mice are considered to be relatively susceptible to secondary (serum amyloid A [SAA]) amyloidosis as well as to senile (AApoAII) amyloidosis.^{S19,S20} It is also considered that stressors (e.g., group housing and infections) have an impact on the incidence of amyloidosis.^{S21,S22}

A urine test was included into the study design for transgenic mice (TgHuA53T) only, that is, proteinuria was monitored weekly from the third month of exposure onward using dipstick urinalysis (Albustix, Siemens, Munich, Germany). It was stated “[p]roteinuria suggests glomerular dysfunction without significant glomerular amyloidosis alterations at this stage.”¹ This is very likely; however, control animals were not monitored. Therefore, these results are useless.

Liver Changes

Boudard *et al.*¹ stated that “[h]istopathological abnormalities in livers from NM-200-exposed mice are provided in detail in Supplementary Table S3 and Supplementary Figure S2”. However, the conclusion of this report is that NM-200 induced renal and liver lesions. It is, therefore, considered necessary to show the results well presented in a main document.

Regarding the liver, it was summarized that NM-200 induced liver inflammation and amyloidosis in C57BL/6 mice.¹ Amyloidosis is, however, a systemic disease. When amyloidosis occurs in kidneys, it likely occurs in livers as well. Nevertheless, it is a normal background lesion in this mouse strain. It occurs spontaneously at high incidences in mice, as discussed previously. The infiltrates and the vacuolation (very likely fat) are very normal background lesions in mice and rats of any strain. The only change that was slightly outstanding is presented in Figure S2f. This case might be considered a secondary infiltration after necrosis, however, is occasionally also observed in control mice as an idiopathic lesion. It was described that occasionally apoptotic bodies occurred and were associated with lymphocytic infiltrate. This, however, can be observed also in control animals from studies under SPF, and are deemed to be part of the normal hepatocellular turnover in mice (own experience: the author of this letter to the editor has been working daily on the microscope for approximately 30 years). Inflammatory cell foci may be, for example, in rats recorded even in young adult control animals at incidences up to 100%.^{S23} The term “necro-inflammatory” lesion for such a condition is not deemed to be adequate. It remains also unclear why an induced liver inflammation was established under uptake of NM-200. In Supplementary Table S3, no real differences are visible between control and treated mice. All illustrations provided for the liver in Supplementary Figure S2 are deemed to be background lesions.

Changes in Transgenic Mice

In the kidneys from transgenic mice, a vacuolar change is presented in Supplementary Figure S4D.¹ There are vacuoles resembling lipid inclusions in mesangial cells.

It is unclear if this is a single glomerulus, and the age of the respective animal is not stated. This change might be consistent with the lipid storage in lysosomes described by Yabuki *et al.*,^{S13} and, hence, would fit glomerular changes under control conditions.

Boudard *et al.*¹ stated that “hepatocytes from mice deleted for α -synuclein displayed steatotic-like cytoplasmic vacuolization (micro- or macro[-]vacuoles similar to those observed in human steatosis), independent of their exposure to SAS NM-200.”¹ Again, fatty change is a common finding in mouse livers.^{S7}

In summary, a low number of transgenic animals were evaluated. It is also questionable if a historical data bank on spontaneous lesions for this mouse strain exists. There were also a few deaths during the study, whereby it is not clear how many exposed and control animals died. The cause of death was not established.

Silica Content in Different Organs

Total silicon was determined in tissues using an Agilent 8800 ICP-QQQ mass spectrometer. The following results were obtained: “In kidney, silica levels were higher in C57BL/6 mice compared with both C57BL/6S mice and controls (statistically significant, $P < 0.01$).”¹ This is not understood, because the relationship to control is not clear. Furthermore, it was stated “Silica deposition in liver followed the order C57BL/6 mice > C57BL/6S mice > control mice, although with a high inter-individual variability. The silica concentration was higher in livers compared with kidneys of C57BL/6 mice.”¹ However, again, it remains unclear what “control” does mean. In the Methods section it is written that “[s]tatistical analyses for tissue silicon content were carried out comparing exposed to control groups by means of the Mann-Whitney U test.”¹ Therefore, one would expect to see control bars, 1 for each control group. With “ $n = 4-6$ for control mice”¹ it remains completely unclear how this fit to $n = 7$ for C57BL6 and $n = 8$ for C57BL6/S as given in the Supplementary Material. How were animals selected for Si determination or what happened with missing values if all animals were to be processed? Without comprehensive information on the data set used for statistical analysis, the statistical significance remains scientifically useless. The same is true for the nonsignificant trend shown in Figure 1A. Furthermore, in Figure 1B, the difference for the amount of Si in kidneys between “control” and C57BL/6S values seems to be on a biologically similar level. Any statistical relevance should also be checked for a biological relevance. This has not been addressed. Considering these limitations of the data there are several further questions to be asked:

- (i) How much Si is normally present in the organs of control animals? With having a permanent natural background of silicon exposure (Si is one of the most common elements on earth and is bound to silica minerals that are omnipresent, e.g., feldspars, plagioclases) and various possibilities of silicon contamination during animal treatment and sample preparation, it is of high relevance to have a sound basis for the natural background of silicon in these organs if conclusions are drawn from a limited database.
- (ii) What could be the source of silicon in organs? With ICP-as being a non-substance-specific analytical method, it is not possible to clarify the source of silicon found in organs. Therefore, it becomes extremely important to have exact information on possible sources of silicon exposure from feed and drinking water. Impurification by Si is absolutely normal due to the presence in Si in the environment. This shows up in high silicon contents in food and feed basis material. The European Food Safety Authority provided an estimate of the silicon content in food: “High levels of silicon are found in foods derived from plants, particularly cereals such as oats (3910–4310 mg/kg dry weight), barley (2610–2720 mg/kg dry weight), white wheat flour (81–103 mg/kg dry weight), or polished rice (55–57 mg/kg dry weight).”^{S24} In drinking water, the silicon level varies from region to region and might be higher in mineral springs. Normal drinking water contains silica at a range from 4.2 to 22.4 mg/L.^{S25} Bottled silicon-rich water contains up to 85 to 90 mg/l silica. However, also beer contains 0.9 to 3.94 mg silicon/100 g.^{S26}
 - It should also be mentioned that rodent diet in many cases contains synthetic amorphous silica as food additive (E 551) for the equal distribution of substance as, for example, vitamins.

CONCLUSION

The applied dosage of 4.8 mg SiO₂/kg body weight per day to mice by drinking water is very low considering background Si level by normal drinking water and diet and cannot be even considered to induce lesions. Based on the presented study design and the findings established, including the silicon analysis in different organs, no effect of Si on the liver and kidney can be stated. All lesions described are within the range of normal expected background alterations in wild-type mice of this strain and age. No control data are available from the type of transgenic mice used.

DISCLOSURE

KW (AnaPath GmbH) works as a consultant for and ND who is a member of the Association of Synthetic

Amorphous Silica Producers (ASASP). JF (PQ Corporation), MK (Wacker Chemie AG), NK and TBS (Evonik Resource Efficiency GmbH), JN (Grace Europe Holding GmbH), J-AS (Solvay), and DS (Pittsburgh Plate Glass Company) produce synthetic amorphous silica.

SUPPLEMENTARY MATERIAL

Supplementary File (PDF)

Supplementary References.

1. Boudard D, Aureli F, Laurent B, et al. Chronic oral exposure to synthetic amorphous silica (NM-200) results in renal and liver lesions in mice. *Kidney Int Rep.* 2019;4:1463–1471.
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Received 6 November 2019; accepted 25 November 2019; published online 10 March 2020

Kidney Int Rep (2020) 5, 550–554; <https://doi.org/10.1016/j.ekir.2019.11.023>

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The Authors Reply: In this response, we reply to the concerns raised by Weber *et al.*¹ on our recently published article (Boudard *et al.* 2019)² that provided evidence that chronic oral exposure to a form of synthetic amorphous silica (SAS) used as the food additive E551 (i.e., precipitated silica as NM-200) results in renal and liver lesions in mice. The letter criticizing our article, authored by an



industry consultant (pathologist) and co-authored by representatives of several SAS manufacturers, claims deficiencies in the study design and other methodological issues that, according to their judgment, would jeopardize the overall conclusions of our study. An itemized reply to their criticism is given hereunder.

STUDY DESIGN

Selection of Strains

Weber *et al.* argue that the C57BL/6 mouse line may not be the first choice for long-term studies because male individuals have been found to develop, at least to a certain extent, liver tumors and renal diseases as their age increases.¹ Of course, functional losses and appearance of neoplastic or non-neoplastic lesions in aging animals are inevitable in all mouse lines, similar to what is observed in humans. The pattern of disease susceptibility with age differs mainly because mice are more prone to cancer^{S1}; however, neoplastic lesions were not the focus and have not been observed in our study. Our selection of C57BL/6 mice, *females* rather than males, was based on more than 20 years of experience in long-term studies using this mouse line. For specialized investigations aiming at comparing normal aging with slow neurodegenerative process, we have developed an ability to deal with aging animals, optimizing and standardizing experimental conditions in which groups of female mice could be followed for 1 to 3 years.^{S2,S3} In our experience, abnormal occurrence of liver or renal lesions in these aging female mice, even in very elderly individuals, can be definitely ruled out. The same holds true for the other mouse line introduced more recently and directly derived from the C57BL/6 line, the C57BL/6S line solely differing for the absence of alpha-synuclein expression.

Our study² confirmed that it is possible to breed these types of animals for 18 months without apparent morbidity and spontaneous deaths in the control groups. The key point that Weber *et al.* fail to appreciate is that the kidney and liver lesions documented in our study were observed solely in exposed animals. This rules out that selection of the mouse line played any role in the finding. That having been said, it would be interesting to reproduce the experiment with other rodent lines and we hope that the scientific community will be encouraged to do so by our study.

General Study Design

Silica Content of Water and Diet

Weber *et al.* complain about the lack of information on the silica content in the drinking water and animal diet