(Working Draft) Comment

on the

Proposal for Harmonised Classification and Labelling for Silver zinc zeolite.

Prepared by EBRC Consulting GmbH

on behalf of the Precious Metals and Rhenium Consortium.

Based on the

Proposal for Harmonised Classification and Labelling

Based on Regulation (EC) No 1272/2008 (CLP Regulation), Annex VI, Part 2

Substance Name: Silver zinc zeolite (Zeolite, LTA framework type, surface modified with silver and zinc ions)

Prepared by Sweden, Version number: 4, Date: 13th April 2015

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Part B.

Introduction

EBRC Consulting GmbH ("EBRC") has been asked by the Precious Metals and Rhenium consortium ("PMRC") to review the CLH proposal on Silver Zinc Zeolite (SZZ) regarding the assessment of the toxicological hazard profile and proposed classification, with a special focus on the potential relevance of some of the conclusion for inorganic silver substances themselves.

EBRC consults PMRC in toxicological aspects in the context of REACH registrations of several inorganic silver substances, such as elemental silver, disilver(I)oxide, silver nitrate, silver chloride, silver bromide, silver iodide, silver(I)sulfate and silver(I)carbonate. In this capacity, EBRC has worked on the toxicological assessment of "silver" since 2006.

It is noted that the toxicological assessment of SZZ is partly based on "read-across", i.e. that certain toxicological properties are not assessed based on substance-specific data on SZZ, but that information and studies on silver substances (e.g. silver chloride or silver acetate) is usede. Based on the potential influence on the classification of inorganic silver substances, this comment aims at providing additional information relevant for the interpretation of effects observed in toxicological studies with silver substances.

Further, for purposes of comparison, reference is also made in this comment to human health data on "pure" (i.e., non-silver substituted) non-fibrous synthetic zeolites, which allows assigning some observed health effects (i.e., in kidneys) to the zeolite moiety itself, with no attributable contribution by the silver component.

We have used the CLH report template for our comments, to facilitate allocation of the comments. Our comments are focused on topics where the discussion impacts "silver" in general. We do not provide in-depth comments on silver-substituted Zeolites themselves, understanding that the Task Force supporting these substances have voiced their own standpoint. We also do not offer comments on physico-chemical properties, nor on the environmental fate or ecotoxicity of "silver"; the respective sections in our comment are therefore left empty.

Part B

SCIENTIFIC EVALUATION OF THE DATA

1 IDENTITY OF THE SUBSTANCE

No comments.

2 MANUFACTURE AND USES

No comments.

3 CLASSIFICATION FOR PHYSICO-CHEMICAL PROPERTIES

No comments.

4 HUMAN HEALTH HAZARD ASSESSMENT

4.1 Toxicokinetics (absorption, metabolism, distribution and elimination)

No comments.

4.2 Acute toxicity

No comments.

4.3 Specific target organ toxicity – single exposure (STOT SE)

No comments.

4.4 Irritation

No comments.

4.5 Corrosivity

No comments.

4.6 Sensitisation

No comments.

4.7 Repeated dose toxicity

We have two comments to this section:

(1) On page 54 of the CLH proposal (1st paragraph) in the description of the rat study 6.4.1(06), a speculation of the author is reiterated that some "alterations in erythropoietic parameters are suggestive of possible zinc toxicity".

To this, we politely note that zinc and zinc compounds were subjected to a thorough EU risk assessment under the ESR regulation (793/93), rapporteur MS being the NL. In short, the findings of this RAR do not support this speculation, and (among others) for example conclude on a human NOAEL of 50 mg Zn/day for an adult human.

We politely suggest to either put the no-effect levels in that RAR into perspective with the administered dose of zinc in this study to substantiate this speculation, or alternatively consider deleting this speculation.

(2) The repeated dose toxicity studies on silver and/or zinc modified zeolites summarised in this chapter report several adverse effects, such as altered pigmentation in some organs and histopathological effects in kidneys.

Since a substantial data base is available for non-silver or zinc-modified zeolites, we believe it would be prudent to consult these in order to assess whether any of these effects already occur when animals are treated with zeolites on their own.

For this purpose, reference is made to section 6 (subsection 6: repeated dose toxicity) of this commentary, where oral and inhalation repeated dose toxicity studies with unmodified zeolites are summarised. In oral repeated dose toxicity studies, adverse effects in the kidneys and urinary bladder have been consistently reported, and the deposition of crystalline material in the kidney and the excretion of this material via the urine is thought to have contributed via mechanical damage in the kidney and bladder to the concurrent epithelial hyperplasia in these organs

4.8 Germ cell mutagenicity (Mutagenicity)

No comments.

4.9 Carcinogenicity

No comments.

4.10 Toxicity for reproduction

4.10.1 Effects on fertility

4.10.1.1 Non-human information

In two-generation reproduction studies with silver-substituted zeolites, adverse effects included deposition of crystalline material in the kidney and epithelial hyperplasia in kidney and bladder. By comparing these effects with those caused by "unmodified" zeolites, it can be assumed that the hydronephrosis observed in F1 animals in the two-generation study with SZZ is due to zeolites per se.

To elaborate on this point, a brief summary assessment of the two-generation reproductive toxicity studies with the biocidal compounds silver zinc zeolite (SZZ) and "silver containing active substance 2" is given below, plus a comparison to data on zeolite itself. For a more detailed toxicological profile, we also make reference to data summarised in section 6 below.

(1) Silver zinc zeolite (SZZ)

Groups of male and female CD Sprague-Dawley rats were administered silver zinc zeolite (SZZ, denoted Zeomic in IIIA 6.8.2-04 / Schroeder, 2002) at dietary levels of 0, 1,000, 6,250 and 12,500 ppm over two generations throughout maturation, mating, gestation and lactation. In the F1 parenteral generation, considerable mortality was seen in the 12,500 ppm group and, therefore, the group was terminated at the end of the premating growth period. The average combined intakes for P and F1 male rats during the premating period were 72, 472 and 984 mg Zeomic/kg bw/d. In the parenteral females the intakes were 87, 548 and 1109 mg Zeomic/kg bw/d. Dose levels for females during gestation were similar to those seen during the premating period and slightly higher during lactation.

Parental mortality was seen at the 6,250 and 12,500 ppm groups. In the 12,500 ppm group, three males died and in the F1 generation, 28/30 males and 23/30 females died. Because of this mortality the F1 12500 ppm group was terminated at completion of the premating period. In the 6250 ppm group, one P male and eight F1 animals (7 males/1 female) died.

The NOAEL for reproductive toxicity/fertility was established at 1,000 ppm Zeomic (approx. 79 mg/kg bw/d). Based on the correction for silver content (6%) and silver ion release (37.4% at pH 4 and 37°C, cf. O´Connor & Woolley, 2010) this NOAEL was converted to a silver ion equivalent of 1.57 mg Ag+/kg bw/d. The only effect on reproductive performance at 6250 ppm (a dose level of approximately 510 mg/kg bw/d or 11.4 mg Ag+ equivalents/kg bw/d) over the two generations was a decrease in live-birth index and increase in stillborn index in the F2 litters.

In the 12,500 ppm group, which received a dose level of approximately 1,047 mg/kg bw/d and only produced one F1 litter, litter size was reduced with a corresponding increase in stillborn pups. No effect of treatment in the P animals at the 6,250 and 12,500 ppm dose levels and F1 animals at 6,250 ppm was evident from estrous cyclicity, reproductive performance, sperm evaluations, or primordial follicle counts (F1 animals only).

Commented [A1]: This is a summary of the study as compiled by EBRC whilst working on the commentary. Possibly, this can be either removed from the final commentary or considerably shortened, because the facts from this study are already summarised by KEMI.

The NOAEL for pup toxicity was 1,000 ppm based on the following findings at 6,250 ppm and higher: reduced pup weights during lactation, macroscopic findings in offspring (decreased thymus size, cardiac enlargement, renal, hepatic and pulmonary pallor), lower brain, spleen and thymus weights at weaning, delayed vaginal opening and preputial separation, and at 12,500 ppm reduced pup survival during lactation in F1 litters. This NOAEL corresponds to 87 mg Zeomic/kg bw/d or 1.95 mg Ag+ equivalents/kg bw/d.

The NOAEL for parental systemic toxicity was considered to be 1,000 ppm based on lower body weights and lower body weight gains in mid and high dose males and high dose F1 animals, lower food consumption in mid and high dose males, haematological changes (increased red blood cells and platelets, and decreases in haemoglobin, haematocrit and other blood parameters), increased cholesterol levels in both sexes, and decreased kidney weights.

Macroscopic changes consisted of organ pigmentation, especially of glandular tissues/organs. The findings were seen mainly in both generations at 6,250 and 12,500 ppm and also in several 1,000 ppm animals. Other changes were noted in the urinary tract at mid and high dose levels and included mild calculi, mild to moderate pelvic dilatation (hydronephrosis), and an increased incidence of mild to moderate cortical surface irregularities which corresponded microscopically to interstitial nephritis and/or infarction. These changes along with distention of the ureters and urinary bladder were considered to have consequences in lower urinary tract obstruction. Based on the reported test substance intake, this NOAEL corresponds to 72 or 87 mg Zeomic/kg bw/d (or to 1.62 or 1.95 mg Ag+ equivalents/kg bw/d) for males and females, respectively.

(2) "silver containing active substance 2" (Doc IIIA, 6.8.2(3))

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This is a summary of the study as compiled by EBRC whilst working on the commentary. Possibly, this can be either removed from the final commentary or considerably shortened, because the facts from this study are already summarised by KEMI.

This study is addressed in the CLH Report in chapter 4.10.3. since it is not a study with SZZ, but instead with a similar, other substance. We have included a presentation of this study here in 4.10.1 of this commentary in order to facilitate a comparison of effects with the two-generation study on SZZ as summarised above.

Groups of male and female Sprague-Dawley Crl:CD(IGS BR) rats were administered "experimental additive number 9823-37" ("silver containing active substance 2" (doc IIIA, 6.8.2(03)) in dietary doses of 1,000, 5,000 and 20,000 ppm (72.5/78.2, 363/400 and 1465/1612 mg/kg bw/d) over two successive generations throughout maturation, mating, gestation and lactation (OECD guideline 416) (Wood & Finn, 1998).

Results (adults):

At 20,000 ppm, there was a reduction in body weight gain for F1 males and females only during maturation. F1 male food consumption values were also lower during maturation. Body weight for F1 females was only reduced during gestation and lactation and there was lower food consumption during these periods.

Reproductive performance for both generations was generally unaffected by treatment. A slight increase in pre-coital times for a small number of animals did not result in an adverse effect upon pregnancy. There were no significant effects upon reproductive organs both macroscopically and microscopically and no effects on semen characteristics or oocyte numbers.

Target organ effects were associated with the pancreas and mesenteric lymph nodes which were related to accumulation of pigment within the tissues, but no adverse histopathological changes were observed. Organ weight analyses gave no consistent effects on the organs evaluated.

At 5,000 ppm, there were no significant effects on adults of both generations during the in life phase of the study. There were no effects on reproductive performance. No post mortem effects were observed for the reproductive organs and no effects on semen analysis were seen. Similar target organ pigment accumulation to that seen at 20,000 ppm was observed for the pancreas and mesenteric lymph nodes of both sexes and generations but at a lower incidence. Inconsistent organ weight differences were observed that were considered unrelated to treatment.

At 1,000 ppm, there were no effects on adults of either generation during the in-life phase of the study. There were no effects on reproductive performance for either generation. Post mortem findings showed no effects on target organs, i.e., no pigmentation of tissues. No effects on reproductive organs were observed.

Results (offspring):

At 20,000 ppm, the live litter size at birth was reduced for the F1 mating phase only, but the subsequent offspring viability was unaffected. There was also a lower body weight for offspring at lactation day 21 for both the F0 and F1 mating phases. There were no effects on offspring physical development or sexual maturation of selected F1 animals. The weight of thymus was reduced in both male and female pups of both generations and was considered a treatment-related effect.

At 5,000 ppm, no effects upon litter size or viability during lactation were found for either generation. Offspring growth, physical development and/or sexual maturation were unaffected by treatment. The weight of thymus was reduced in either males or females, but not both, for both generations. The actual recorded values were comparable to historical control values and therefore considered of limited toxicological relevance by the authors.

At 1,000 ppm, there were no effects upon litter size or viability during lactation for either generation. Offspring growth, physical development and/or sexual maturation were unaffected by treatment. Post mortem organ weights were unaffected by treatment.

Conclusions:

At 20,000 ppm “silver containing active substance 2”, treatment-related effects on the in life phase of adults were observed for the F1 generation adults only. There were no significant effects on reproductive organs or reproductive performance at any dose level. There was a reduction in live litter size for the F1 mating phase and a reduction in offspring body weight at lactation day 21 for both generations. Reduced thymus weights were seen in the offspring of both generations at the high dose. Pigmentation was observed at the mid and high dose in the pancreas and mesenteric lymph nodes which was not associated with pathological changes. At 5,000 ppm there were no significant toxic effects on adults. Pigmentation was observed in the same tissues as at 20,000 ppm.

Therefore, the NOAEL for reproductive toxicity/fertility of the parent and F1 generation was established at 5,000 ppm based on a reduced litter size in high dose F2 animals and a reduction in offspring body weight at lactation day 21 for both generations. This NOAEL corresponds to 400 mg/kg bw/d of the “silver containing active substance 2” or to a silver ion equivalent of 12.3 mg Ag+/kg bw/d after correction for silver content (10%) and silver ion release (30.7% at pH 4 and 37°C; O´Connor & Woolley, 2010). The LOAEL was 1612 mg/kg bw/d or 40 mg silver ion equivalents/kg bw/d.

The NOAEL for pup toxicity was 1,000 ppm based on reduced thymus weights in high dose F1 and F2 pups and in male mid dose F1 pups. Based on the lowest reported substance intake in females during premating, this corresponds to 78 mg "silver containing active substance 2"/kg bw or 2.4 mg Ag+ equivalents/kg bw/d (F0).

The NOAEL for parental systemic toxicity was considered to be 1,000 ppm based on organ pigmentation (pancreas, mesenteric lymph nodes in both sexes and generations) and organ weight changes at higher doses. Based on the lowest reported test substance intake during premating, this NOAEL corresponds to 72.5 mg "silver containing active substance 2"/kg bw/d or to 2.2 mg Ag+ equivalents/kg bw/d (F0 males). The corresponding LOAEL is 363 mg active substance 2/kg bw/d and approximately 11 mg Ag+ equivalents/kg bw/d (F0 males).

(3) Summary of NOAELs:

SZZ

The NOAEL for reproductive toxicity/fertility was determined at 1,000 ppm Zeomic (approx. 79 mg/kg bw/d which corresponds to a silver ion equivalent of 1.57 mg Ag+/kg bw/d). The only effect at the next dose level of 6,250 ppm (LOAEL, approximately 510 mg/kg bw/d or 11.4 mg Ag+/kg bw/d) over the two generations was a decrease in live-birth index and increase in stillborn index in the F2 litters. There were no statistically significant or clearly dose-related effects on fertility parameters.

The NOAEL for pup toxicity was 1,000 ppm corresponding to 87 mg Zeomic/kg bw/d or 1.95 mg Ag+ equivalents/kg bw/d. It was based on the following findings at 6,250 and 12,500 ppm: reduced pup weights, macroscopic findings (decreased thymus size (thymic atrophy), cardiac enlargement, renal, hepatic and pulmonary pallor), lower brain, spleen and thymus weights at weaning, and at 12,500 ppm reduced pup survival during lactation in F1 litters.

The NOAEL for parental systemic toxicity was considered to be 1,000 ppm based on lower body weights and lower weight gains in mid and high dose males and high dose F1 animals, and on changes in the kidneys at mid and high doses including calculi formation and mild to moderate hydronephrosis. Based on the reported substance intake, this NOAEL corresponds to 72 or 87 mg Zeomic/kg bw/d (1.62 or 1.95 mg Ag+ equivalents/kg bw/d) for males and females, respectively.

"Silver containing active substance 2"

The NOAEL for reproductive toxicity/fertility of the parent and F1 generation was 5,000 ppm "silver containing active substance 2" which corresponds to 400 mg/kg bw/d or to a silver ion equivalent of 12.3 mg Ag+/kg bw/d). This NOAEL is based on a reduced litter size in high dose F2 animals and a reduction in offspring body weight at lactation day 21 for both generations at the 20000 ppm dose level representing the LOAEL (1612 mg/kg bw/d or 40 mg Ag+/kg bw/d.

The NOAEL for pup toxicity was 1,000 ppm corresponding to 78 mg /kg bw or 2.4 mg Ag+ equivalents/kg bw/d (F0). It was based on reduced thymus weights in high dose F1 and F2 pups and in male mid dose F1 pups representing the LOAEL of 400 mg/kg bw/d or 12.3 mg Ag+ equivalents/kg bw/d.

The NOAEL for parental systemic toxicity was 1,000 ppm based on organ pigmentation (pancreas, mesenteric lymph nodes in both sexes and generations) and organ weight changes at higher doses.

This NOAEL corresponds to 72.5 mg /kg bw/d or to 2.2 mg Ag+ equivalents/kg bw/d (F0 males). The corresponding LOAEL was 363 mg /kg bw/d or approximately 11 mg Ag+ equivalents/kg bw/d (F0 males).

(4) References:

Fruijtier-Pölloth C (2009) The safety of synthetic zeolites used in detergents. Arch Toxicol 83, 23-35

Gloxhuber C, Potokar M, Pittermann W, Wallat S, Bartnik F, Reuter H, Braig S (1983) Zeolite A – A phosphate substitute for detergents: Toxicological investigation. Food Chem Tox 21, 209-220

O´Connor BJ, Woolley SM (2010) Release of silver into aqueous media under different conditions. Harlan Laboratories Ltd, Project number: 2560/0001

OECD (2006) SIDS Initial assessment profile: Crystalline, non-fibrous zeolites. 23nd SIDS Initial Assessment Meeting, 17-20 October 2006, Jeju, South Korea

Schroeder RE (2002) A dietary two-generation reproduction and fertility study of Zeomic in rats. MPI Research, Inc., Laboratory study number: 892-002

Wood E, Finn JP (1998) Experimental additive number 9823-37: Dietary two-generation reproduction study in the rat. SPL Project number: 656/082

4.10.1.2 Human information

No comments.

4.10.2 Developmental toxicity

No comments, except for those rendered to section 4.10.3 below on the use and interpretation of "read-across" information on silver substances other than silver-modified zeolites.

4.10.3 Other relevant information

The CLH Report for silver zinc zeolite (Version 4, 13 April 2015) in its proposed classification as "Reprotoxic category 1B (H360D)" refers to a publication by Shavlovski et al. (1995) entitled "Embryotoxicity of silver ions is diminished by ceruloplasmin – further evidence for its role in the transport of copper".

In a first instance, the comment rendered below provides an argumentation that the embryotoxic effects of silver chloride in rats following dietary administration of a very high dose of 188 mg Ag/kg bw during the entire period of gestation (Shavlovski et al., 1995) can be considered not to be a direct effect of silver ions on embryogenesis, but instead represent a "secondary non-specific consequence" of the disruption of maternal copper homeostasis resulting in copper deficiency.

Further, recent data (Boudreau, 2012) clearly indicate that oral exposure of rats to high doses of an inorganic silver substance (silver acetate) caused a massive shift in intestinal microbiota resulting in

severe gastroenteritis, and thus contributed further to disruption of the homeostasis in the intestinal tract.

Finally, an extended discussion of the relevance of ceruloplasmin and the influence of administration with either silver and/or copper is provided with the objective to demonstrate that silver itself has no direct developmental toxicity potential.

Overall, the available mechanistic information supports the assumption of a "secondary non-specific mechanisms" involving disruption of maternal homeostasis, thus not justifying a classification for developmental toxicity Category 1B or Category 2.

(1) Discussion of the study by Shavlovski (1995)

Introduction

In the CLH Report for silver zinc zeolite (Version 4, 13 April 2015), decisive reference is made with respect to the proposed classification for Reprotoxic category 1B (H360D) to a publication by Shavlovski et al. (1995) entitled "Embryotoxicity of silver ions is diminished by ceruloplasmin – further evidence for its role in the transport of copper".

That study with silver chloride is then used in sections 4.10.4 Summary and discussion of reproductive toxicity, 4.10.5 Comparison with criteria and 4.10.6 Conclusions on classification and labelling as supporting data cited as "clearly demonstrating developmental toxicity in rats".

With respect to the diverse functions of copper-containing ceruloplasmin (cf. Hellman & Gitlin, 2002, Nevitt et al., 2012) it was shown that ceruloplasmin which is the major copper-containing protein in the blood is the main normal source of copper for placenta and fetus (Lee et al., 1993) thus being of essential function for the development of offspring.

The study by Shavlovski et al. (1995) was conducted for the purpose of understanding the mechanism of ceruloplasmin-associated copper deficiency upon embryogenesis.

Influence of silver salts on copper status in rodents

Studies in rats

Shavlovski et al. (1995) published a paper entitled "Embryotoxicity of silver ions is diminished by ceruloplasmin – further evidence for its role in the transport of copper".

Previous studies by the same group of authors (Pribyl et al., 1989) had shown that feeding rats a silver nitrate-supplemented diet (60 mg $AgNO_3$/kg bw/d, equivalent to 38 mg of silver/kg bw/d) for more than 7 days decreased the ceruloplasmin-dependent oxidase activity in the blood to about 20%. Further treatment for up to 16 days led to a complete loss of oxidase activity in blood and resulted in the formation of a structurally modified ceruloplasmin as indicated by substitution of copper atoms for four silver atoms and loss of the characteristic absorption band at 610 nm.

Shavlovski et al. (1995) administered sparingly soluble silver chloride (50 mg/animal, corresponding to about 250 mg/kg bw/d which is equal to 188 mg of silver/kg bw/d) in diet to 5 inbred albino female rats during days 7-15 of gestation. The AgCl treatment during the period of organogenesis did not affect the development of embryos. Pre-implantation and post-implantation

losses, the number and the body weight of fetuses corresponded to those of the controls (20 animals). No external abnormalities appeared on day 20 of gestation.

A group of 20 rats was also administered the same high amount of AgCl throughout the entire period of gestation (gd 1-20). This treatment resulted in a considerably increased incidence of post-implantation losses of 36.0% compared to that of 9.6% of the control group, whereas the pre-implantation losses were not affected. The number of live fetuses and the fetal weight were decreased by 81% and 22%, respectively. Five out of 145 embryos (3.4%) showed visible abnormalities. The number of embryos having visceral damages was considerably higher in this trial group. The incidences of hydronephrosis (31%) and cryptorchidism (35%) increased considerably compared to controls (5.3% and 1.3%, respectively). In addition, the incidence of haemorrhages increased. Moreover, all of the totally little number of newborns died within 24h of birth.

AgCl administration throughout the entire gestational period resulted in a reduced copper content of about 65% in maternal tissues (liver, heart, and kidney, cf. Table 3) and the absence of copper in placenta, embryonic tissues and maternal serum which correlated with the disappearance of oxidase activity from circulation (data not shown). Simultaneous i.p. injection of purified human ceruloplasmin into pregnant rats treated with AgCl especially during the second half of gestation caused a considerable reduction of adverse effects in dams and in the embryonic development including lower mortality of newborns. On the other hand, simultaneous introduction of the copper chelator penicillamine during the whole term increased the post-implantation loss to totally 79% thus providing additional substance to the argument of an involvement of copper deficiency to the toxicity caused by AgCl. Based on these findings, the authors concluded on an embryo-/fetotoxic effect of AgCl which is caused by the ability of silver to interfere with copper metabolism, in particular by altering the copper-transporting function of ceruloplasmin and, consequently, resulting in a copper deficiency in the developing tissues of embryos.

The limitations of the study by Shavlovski et al. to serve as substitute for a prenatal developmental toxicity study (OECD TG 414) have already been pointed out in detail in the expert statements on reproductive toxicity of silver zinc zeolite prepared by C. Price & R. Tyl (007), St. Barton (006) and V. Mostert (003), and are therefore only briefly addressed here:

a proper appraisal of the effects described in the Shavlovski et al. paper is impaired due to lack of data relating to potential maternal toxicity (body weight change, body weight gain, food intake, water consumption and clinical signs). Actual dietary intake was not reported. Maternal effects were only mentioned in the Abstract ("… AgCl did not cause alterations of their physiological functions, ...") without giving any detailed information in the paper. In addition, no data on litter size and individual fetal body weights are presented. Thus, an interpretation of effects in connection with litter size and fetal weight data are not possible. Because only one dose was tested no dose-response relationship is available, thus defining NOAELs for both maternal toxicity and embryo-fetal toxicity is not possible.

However, the reported loss of the copper-dependent oxidase activity and the lack of copper in the maternal blood serum demonstrate the appearance of possibly adverse effects in dams. In addition, the copper content in maternal tissues (liver, heart, and kidney) was reduced to 65% indicating a distinct copper deficiency. Moreover, copper was not detectable in the placenta.

Taking together the results from both studies, treatment of rats with doses of about 38 mg of silver/kg bw ($AgNO_3$) or estimated 188 mg of silver/kg bw (AgCl) for 16–20 days resulted obviously in formation of a structurally modified ceruloplasmin in which 4 copper atoms were

replaced by silver atoms. The consequence of this is an impairment of the copper transport function of ceruloplasmin and the deactivation of its copper-dependent oxidase activity in circulation.

Other studies on copper status in rats

To permit a more comprehensive assessment of the adverse effects on embryonal and fetal development following the repeated exposure of silver chloride to female rats, further studies on the influence of repeated administration of silver salts on the copper status in rats and mice were taken into consideration. Although involving non-pregnant animals, it can nevertheless be assumed that the biochemical alterations caused by silver in non-pregnant animals will also proceed under specific conditions of gestation.

Changes of copper concentrations in rat tissues after silver administration as well as in the status of serum ceruloplasmin have been shown by Hirasawa et al. (1994). Fischer F344 rats (6 males per group) received i.p. about 1 mg of silver/kg bw as silver nitrate for 6 successive days. The administered silver deposited in all the tissues examined, particularly in the pancreas, followed in that order in spleen = liver, > kidney > lung >heart. Hepatic and pancreatic copper concentrations were significantly increased after silver administration, whereas renal and serum copper concentrations were significantly decreased. These results suggest that silver causes a re-distribution of tissue copper in the rat body. Serum copper concentration and ceruloplasmin oxidase activity were decreased to 60% and 25% of the control values, respectively. Immunoblotting of serum ceruloplasmin of silver-treated groups showed only a slight decrease in its concentration in serum suggesting that most of the ceruloplasmin was still present but as an oxidase inactive form. Simultaneous i.p. injection of about 3 mg of zinc (as zinc sulfate) for 6 days did not reverse the silver-provoked decreases in serum copper concentration as well as serum ceruloplasmin oxidase activity levels. No consistent changes in iron concentration were observed in any of the tissues examined after silver and/or zinc administration. In conclusion, the results indicate that silver administration to rats caused a disturbance of copper metabolism and of ceruloplasmin metabolism, but zinc did not protect against such changes.

Hirasawa et al. (1997) purified an enzymatically inactive ceruloplasmin from the serum of Fischer F344 rats treated i.p. with 1 mg of silver/kg bw as silver nitrate for 6 days. Silver treatment resulted in decreases of copper concentration and ceruloplasmin oxidase activity in serum to 60% and 10% of the control values, respectively, and a marked accumulation of silver in serum. The metal contents of the Ag-modified ceruloplasmin were estimated as about 0.8 atoms of silver and 4.2 atoms of copper per molecule compared to 5.9 atoms of copper for ceruloplasmin from control rats. The Ag-modified ceruloplasmin showed neither a blue colour (characteristic for native protein, cf. Hellman & Gitlin, 2002) nor an EPR signal as characteristics of normal holo-ceruloplasmin. Thus, silver binding to native holo-ceruloplasmin resulted in a loss of one or two of the copper atoms essential for the oxidase activity.

Change of copper status in blood serum (copper concentration, ceruloplasmin oxidase activity, and ceruloplasmin protein content) of adult Wister rats and C57B1 mice was caused by feeding AgCl-containing diet (50 mg/kg bw/d, equal to 37.6 mg of silver/kg bw/d) for 4 weeks (Ilyechova et al., 2011). In rats, no ceruloplasmin oxidase activity was detectable in serum at that time. Simultaneously, copper concentration in rat blood serum was decreased by 90%, while in mice the copper concentration decreased only by 60%, but serum oxidase activity disappeared after one week on Ag-diet. The ceruloplasmin content in blood sera of Ag-treated animals remained unchanged as evidenced by immunoblotting. Ceruloplasmin existed as two protein forms with different electrophoretic mobility. One of them corresponded by electrophoretic mobility to the holo-

ceruloplasmin, while the other one with lower mobility was considered the apo-ceruloplasmin. Ag-containing ceruloplasmin purified from blood serum of Ag-treated rats contained a molar ratio [Ag] : [Cu] of 4.5 :1 and was shown to exist in a considerably misfolded tertiary/secondary structure of the protein as shown by CD spectra and calorimetric measurements.

Reverse transcription PCR analysis indicated the same levels of ceruloplasmin mRNA in liver of both control and Ag-mice. The same results were reported for Ag-treated rats. Thus, the activity of the ceruloplasmin gene was not affected neither in mice nor rats by Ag-feeding. Recovery of the copper status in rats and mice, fed with Ag-diet for 4 weeks (no oxidase activity in serum), was tested after single i.p. or p.o. injection of CuSO4 (2.6 mg Cu/kg bw) under continuing the Ag-feeding. The Cu2+ injection caused a significant increase of the oxidase activity in the serum of both species starting after 20 min already and recovering to control levels after 4h which was accompanied by rapid insertion of copper into newly synthesized ceruloplasmin in liver. No significant differences between i.p. or p.o. modes of copper injection were observed. The recovered copper status persisted for 3 days under continuing Ag-diet. Direct addition of CuSO4 to oxidase inactive serum of Ag-treated animals did not lead to recovery of oxidase activity. Thus, the restored oxidase activity is not the result of an exchange of silver in the existing Ag-modified ceruloplasmin by copper but rather the result of de novo synthesis of holo-ceruloplasmin due to the availability of Cu2+ ions which compensate for the copper lack provoked by silver treatment of animals.

Taken together, the results of that study indicate that dietary silver treatment of rats and mice (37.6 mg of silver/kg bw/d) for 4 weeks caused disappearance of the ceruloplasmin-dependent oxidase activity and reduction in copper content in serum indicating serum copper deficiency. However, ceruloplasmin gene expression in liver was not affected. Single injection of CuSO4 (2.6 mg Cu/kg bw i.p. or p.o.) to Ag-treated animals resulted in recovery of the oxidase activity of serum within 4 h in both species presumably due to de novo synthesis of copper-containing ceruloplasmin.

Other studies on copper status in mice

In another study, C57B1 mice (5 animals per group) received in diet 50 mg AgCl/kg bw/d (equal to 37.6 mg of silver/kg bw/d) for one, two or three weeks to evaluate the rate of development of blood serum copper deficiency (Zatulovskiy et al., 2012). After silver chloride treatment for 3 weeks one group was given a standard diet for 14 days to study the effect of silver removal on serum copper status which was characterized by ceruloplasmin oxidase activity, ceruloplasmin protein concentration and copper content. Serum oxidase activity was not detected after one week of Ag-diet and remained undetectable during the entire period of Ag-diet. After the 3rd day following silver removal from the diet, the ceruloplasmin oxidase activity was progressively restored. Serum copper concentrations changed in accordance with oxidase activity, i.e., they decreased to about 30% after three weeks and increased to the normal value following Ag-free diet. The ceruloplasmin concentration in serum of Ag-mice treated for 3 weeks was comparable to that of control mice. Immunoblotting showed that ceruloplasmin was present as two protein forms with different electrophoretic mobility. One of them corresponded by electrophoretic mobility to the holo-ceruloplasmin, while the other one had a lower mobility. Neither of them, however, displayed oxidase activity.

The Ag-treatment did not change the expression of some genes involved in copper transport (Ctr1 and ATP7B) and of intracellular copper enzymes (superoxide dismutase 1 (SOD1) and cytochrome c oxidase (COX)) as determined by semi-quantitative reverse transcription PCR of total RNA fraction isolated from livers of control and Ag-mice treated for 3 weeks. Similar results were

obtained at the protein level, i.e., Ag-diet did not alter the protein level of Ctr1, ATP7B, SOD1, and COX (Cox4i1 subunit). The intracellular distribution and activity of SOD1 were also unchanged. As well, the rates of oxygen consumption by mitochondria isolated from liver of Ag-treated mice and control mice were identical. Together these data indicate that dietary silver treatment for three weeks does not affect the copper homeostasis in liver. The copper status in serum was clearly restored in Ag-treated mice within three days after the removal of silver from the diet.

In summary, the results indicate that silver administration to mice (37.6 mg of silver/kg bw/d) for three weeks caused a reduction in the ceruloplasmin-dependent oxidase activity and copper content in serum indicating a serum copper deficiency. However, no changes in ceruloplasmin gene expression in liver occurred. In addition, the SOD1 and COX activity were not decreased. Also Ctr1 and ATP7B gene expression and level of these proteins were not affected. Apparently, copper homeostasis in liver cells was not disturbed.

Summary of findings

The alterations in serum copper level and ceruloplasmin oxidase activity in rats after i.p. administration of 1 mg Ag/kg bw for 6 days (Hirasawa et al. 1994, 1997) are qualitatively similar to those reported by the group of Shavlovski (Pribyl et al., 1989, Shavlovski et al., 1995) and recently by Ilyechova et al. (2011). However, there are also very distinct quantitative differences:

Treatment of rats with 1 mg Ag/kg bw for 6 days (as AgNO3) resulted in decreased copper content and oxidase activity in serum to 60% and 10%, respectively, and formation of a modified ceruloplasmin containing about 0.8 Ag atoms and 4.2 Cu atoms in contrast to 5.9 Cu atoms in native holo-ceruloplasmin from control rats (Hirasawa et al., 1994).

However, feeding rats a higher amount of 38 mg Ag/kg bw/d for 16 days (as AgNO3) resulted in a total loss of oxidase activity and formation of another structurally modified ceruloplasmin form containing 4 Ag atoms instead of copper (Pribyl et al., 1989).

Nearly identical results were reported recently (Ilyechova et al., 2011). Feeding rats a Ag-diet with 37.6 mg Ag/kg bw/d (as AgCl) for 4 weeks resulted in no detectable oxidase activity and a reduced copper concentration by 90% in serum, while the ceruloplasmin content in serum remained unchanged as evidenced by immunoblotting. The Ag-containing ceruloplasmin purified from serum of Ag-treated rats contained a molar ratio Ag:Cu of 4.5:1 and was shown to exist in a drastically misfolded tertiary/secondary structure. However, ceruloplasmin gene expression in liver was not affected by Ag feeding as measured by RT PCR analysis. Single injection of CuSO4 (2.6 mg Cu2+/kg bw) to Ag-treated rats and mice resulted in recovery of the oxidase activity of serum within 4h in both species presumably due to de novo synthesis of copper-containing ceruloplasmin.

Feeding female rats a very high dose of estimated 188 mg Ag/kg bw/d (as AgCl) during entire gestational period (gd 1-20) resulted in the absence of copper and oxidase activity in serum and a markedly reduced copper content in liver, heart, and kidney, while copper was completely absent in placenta and embryonic tissues (Shavlovski et al., 1995). Thus, besides the Ag-caused alterations in serum copper status the markedly reduced copper content in maternal tissues has to be considered as indication of strongly disrupted copper homeostasis in dams, obviously without any clinical signs of copper deficiency or maternal toxicity as stated by the authors. Therefore, the high level of embryolethality and the death of all newborn within 1 day can be attributed to the severe copper deficiency. The even higher embryolethality of 79% due to simultaneous introduction of the copper chelator penicillamine during the whole term gives further support to copper deficiency as cause of AgCl-mediated developmental toxicity. Otherwise, there were no developmental effects in the

offspring of female rats treated with 188 mg Ag/kg bw/d in diet only during days 7-15 of gestation. Obviously, the shorter AgCl treatment during the period of organogenesis did not yet result in systemic copper deficiency thus allowing undisturbed embryo-fetal development.

A 3-week feeding study in mice showed that AgCl diet corresponding to 37.6 mg Ag/kg bw/d caused a reduction in the oxidase activity and copper content in serum indicating likewise a serum copper deficiency (Zatulovskiy et al., 2012). Again, as in rats, no changes in ceruloplasmin gene expression in liver occurred. In addition, the SOD1 and COX activity were not decreased. Also Ctr1 and ATP7B gene expression and level of these proteins were not affected. Apparently, copper metabolism in liver cells was not disturbed. The serum copper status (Cu content and oxidase activity) was clearly restored in the Ag-treated mice within three days after the removal of silver from diet.

In conclusion, the embryotoxic effect of silver chloride in rats following dietary administration of a very high dose of 188 mg Ag/kg bw during the entire period of gestation (Shavlovski et al., 1995) cannot be considered a direct effect of silver ions on the embryogenesis, but to represent a secondary non-specific consequence of the disruption of maternal copper homeostasis in different tissues such as liver, kidney, heart, and placenta resulting in a severe copper deficiency. In addition, the high silver concentrations occurring in circulation are in accordance with formation of Ag-modified ceruloplasmin containing 4 Ag atoms instead of Cu atoms with an irreversibly misfolded structure resulting in a functionally inactive ceruloplasmin which lacks enzymatic activity and copper transport function.

Silver-associated impairment of copper homeostasis and recovery therefrom

Administration of silver doses of 188 mg Ag/kg bw to female rats throughout the entire gestational period resulted in a reduced copper content of about 65% in liver, heart and kidney and the absence of copper in placenta, embryonic tissues and maternal serum which correlated with the disappearance of oxidase activity from circulation (Shavlovski et al., 1995). Thus, it can be assumed that the detrimental effects in the development of fetuses and newborns occurring after exposure of dams to that high silver dose during entire gestation are the result of a disruption of systemic copper homeostasis in dams which is accompanied by formation of a functionally inactive silver-containing ceruloplasmin making impossible transfer of copper to the developing embryo/fetus.

There is substantial evidence that offspring born to copper-deficient dams suffered from developmental abnormalities due to direct impairment of intracellular copper-containing enzymes (cytochrome c oxidase, lysyl oxidase) and showed a significant reduced 24h survival time (cf. Gambling et al., 2011).

In the same study of Shavlovski et al. (1995), no developmental effects were found in the offspring of female rats treated only during days 7-15 of gestation with the equal dose of 188 mg Ag/kg bw. Thus, administration of silver ions during the period of organogenesis did not lead to embryo/fetal toxicity. Obviously, the shorter AgCl treatment did not yet result in a systemic copper deficiency thus allowing undisturbed embryo-fetal development.

It may also be speculated that the observed diminishment of adverse effects in dams and in the embryonic development including lower mortality of newborn due to simultaneous i.p. injections of

purified human ceruloplasmin into pregnant rats fed with AgCl during the entire gestation could be effected by the de novo synthesis of copper-containing ceruloplasmin because it can be presumed that the injected human ceruloplasmin was acting as an external source of copper.

The maintained functional integrity of the ceruloplasmin protein synthesis machinery in rats or mice after feeding diets with 37.6 mg Ag/kg bw/d (as AgCl) for 4 weeks was demonstrated unambiguously by single addition of copper sulfate (2.6 mg Cu2+/kg bw) resulting in formation of an enzymatically active ceruloplasmin (Ilyechova et al., 2011).

There is evidence in the literature, that the extent to which copper deficiency affects pregnancy outcome is very much dependent on the degree of copper limitation. Severe copper deficiency can lead to reproductive failure, early embryonic death and gross structural malformations in the fetus, while moderate or mild copper deficiency has little effect on either number of live births and neonatal weight (cf. Gambling et al., 2011). For the fetus these damaging effects can become apparent before, or even in the absence, of any clinical signs of deficiency in the mother. The range and extent of detrimental effects seen in the developing fetus is dependent on the severity of the deficiency, whether it occurs only for a single "micronutrient", i.e., copper, and the gestational age at which the deficiency occurs.

In the case of the Shavlovski et al. study, accordingly, the detrimental effects with late post-implantation deaths and complete pup mortality after exposure of dams to estimated high silver doses of 188 mg Ag/kg bw/d during entire gestational period can be attributed to result from a severe maternal copper deficiency obviously without of any clinical signs of deficiency or maternal toxicity.

Copper uptake and metabolism

Intracellular copper concentrations are tightly regulated by copper transporter (Ctr) proteins and copper-transporting ATPases (ATP7A and ATP7B; cf. Nevitt et al., 2012). Dietary copper (Cu2+) is reduced to Cu+ and transported into enterocytes primarily via the so-called high-affinity copper transporter 1 (Ctr1) localized on the apical membrane of enterocytes within the lumen of the intestine. Silver is a potent inhibitor of copper uptake via Ctr1 as shown by metal competition experiments in a human embryonic kidney (Hek293) cell line suggesting that Ctr1 may also function in importing silver into cells (Lee et al., 2002). Thus, it can be expected that in the presence of silver salts the import of copper by enterocytes is diminished due to the concomitant import of Ag resulting in copper deficiency within these cells leading to an impairment of intracellular copper-dependent processes and to a decreased enterocyte copper efflux thus lowering the copper levels in blood for further distribution to tissues.

Tracer studies in rats have shown that radioactive copper entering the blood initially binds to albumin and transcuprein forming the "exchangeable copper pool" (cf. Hanson et al., 2001; Donley et al., 2002). Radioactive copper transported mainly by transcuprein then rapidly leaves the blood for the liver and kidney.

Lower copper concentrations in blood are of special importance to the liver, which is the main storage organ for copper and fulfils the decisive function in the regulation of systemic copper homeostasis, especially incorporation of copper into ceruloplasmin and other Cu-dependent proteins (Nevitt et al., 2012). With respect to ceruloplasmin synthesis, the hepatic copper pool is not rate-limiting under normal circumstances, as the serum ceruloplasmin level increases rapidly during

infection, trauma, and three- to fourfold in pregnancy (cf. Hellman & Gitlin, 2002). However, a decrease in the hepatic copper pool, e.g. as occurs in nutritional copper deficiency, results in a marked decrease in serum ceruloplasmin.

Tracer studies in female rats with radioactive silver (110Ag, as AgNO3) have shown that the transport and distribution of silver resemble those for copper in some aspects, particularly with regard to its rapid accumulation in liver (Hanson et al., 2001). However, silver transport in plasma was mainly carried out by α1-macroglobulin. A small proportion of silver was also incorporated into ceruloplasmin. No association of radioactive silver to albumin and transcuprein was detected indicating the carrier function of another macroglobulin than that involved in copper transport.

Overall conclusions

The available data on the impairment of copper status in rats and mice by silver salts result in the conclusion that the embryotoxic effects of silver chloride in rats following dietary administration of a very high dose of 188 mg Ag/kg bw during the entire period of gestation (Shavlovski et al., 1995) have to be considered not as a direct effect of silver ions on the embryogenesis, but instead to represent a "secondary non-specific consequence" of the disruption of maternal copper homeostasis resulting in a copper deficiency.

The disturbed copper homeostasis in dams is accompanied by formation of a silver-modified, functionally inactive ceruloplasmin with 4 silver atoms lacking copper binding function and oxidase activity. Thereby, the availability of copper to the fetus is limited. As a consequence of this deficiency, the development of the fetus and also pup survival are impaired.

Based on the available mechanistic information, the effects observed in developmental toxicity studies shouldbe considered as a secondary consequence of severe maternal toxicity involving a shift in enterobacterial populations, gastroenteritis and perturbed maternal homeostasis. This should therefore be considered a "secondary non-specific mechanism", and not a direct effect of inorganic silver substances on fetal development.

(2) Disruption of intestinal microbiota by high oral dosing with inorganic silver substances

Apart from interference with copper metabolism, high oral dosing with inorganic silver substances also severely impairs intestinal microbiota, leading to gastrointestinal disruption which in turn additionally interferes with the uptake of essential micronutrients, as demonstrated in the following recently available information:

Preliminary results of an experimentally completed subchronic (90-day) study in rats conducted at National Center for Toxicological Research (NCTR) within the National Toxicology Program have shown that oral administration of silver acetate at doses of 200 and 400 mg/kg bw/d resulted in significant decreases in body weight gain and an increased morbidity at 400 mg/kg bw/d caused by severe gastroenteritis. No overt dose-response pathologies were observed for silver nanoparticles (Boudreau, 2012).

As published by Williams et al. (2014), it was investigated how the endogeneous microbial community (microbiota) of the rat intestine was affected during treatment with silver acetate and silver nanoparticles (AgNP). The focus of the analyses was directed to the two predominant bacterial phyla *Firmicutes* and *Bacteroidetes* constituting more than 90% of the microbiota in the intestine. Three genera out of them (*Bacteroides*, *Lactobacillus* and *Bifidobacterium*) and also the

family *Enterobacteria* that includes, along with many harmless symbionts, many of the more familiar Gram-negative pathogens were examined by real-time PCR analyses. In addition, intestinal mucosa-associated immune responses were measured at the level of mucosal protective layer (MUC2 and MUC3), pathogen-associated molecular patterns (Toll like receptors 2 and 4; and NOD2), regulatory molecules (FoxP3 and GRP43) and inflammatory cytokines (interleukin 10 (IL-10) and transforming growth factor ß (TGF-ß).

Silver acetate (AgAc) was administered at doses of 100, 200 and 400 mg/kg bw/d by oral gavage to male and female Sprague-Dawley rats (10 rats/sex/group) twice daily, 7d/week, for 13 weeks. The selected doses of AgAc provided approximately 64, 128 and 192 mg silver/kg bw/d on a mass basis. Effects of silver acetate were compared to those of AgNP with discrete sizes (10, 75 and 110nm) gavaged at doses of 9, 18 and 36 mg/kg bw/d under the same time conditions.

Culture-based analysis of ileal mucosa-associated bacterial populations showed a size- and dose-dependent antimicrobial effect of AgNP. The pattern of results indicated greater antimicrobial activity (measured as CFU) with decreasing nanoparticle size. The dose effect (gavaged with the same size AgNP) was significant for 110nm AgNP in males, whereas in females 75nm and 110nm AgNP showed dose-dependent responses. The antimicrobial activity of rats gavaged with 100 mg/kg bw/d AgAc (equal to 64 mg Ag/kg bw/d) was comparable to that given lower dose of 10nm AgNP (9 mg Ag/mg bw/d). All male rats (10/10) and eight female rats gavaged with 400 mg/kg bw AgAc were moribund. Most of the animals dosed with 200 mg/kg bw had severe gastroenteritis.

Taken together, chronic exposure of rats to AgNP and AgAc apparently resulted in the killing of some bacterial populations in the rat ileum. Therefore, DNA-based microbiota analysis was used to determine how the major bacterial phyla were affected during silver treatment. The examination indicated that administration of low dose AgAc (100 mg/kg bw/d) altered the ratio of *Firmicutes* and *Bacteroidetes* in the ileal mucosa of both male and female rats from about 90:10 (controls) to 65:35 indicating a shift in the bacterial subpopulations representing the two phyla which could be due to changes in the subpopulations of any of the members of these two phyla.

Relative expression of genes of some beneficial bacterial genera, e.g., *Bacteroides*, *Lactobacillus* and *Bifidobacterium* genera, in both male and female rats showed decreased proportions of the *Lactobacillus* population. The expression of *Bifidobacterium* genus was completely lacking in the AgAc-treated male rats. The reduction of Gram-positive bacteria (belonging to *Lactobacillus* and *Bifidobacterium* genus) caused by low doses of AgAc and AgNP was thought to be due to prolonged interaction of silver ions with charged molecules in the multilayer cell wall of Gram-positive bacteria.

There was a highly significant increase in the expression of *Enterobacteria* family-specific genes including many of the more familiar Gram-negative pathogens, such as *Salmonella*, *Escherichia coli*, *Yersinia*, *Klebsiella* and *Shigella*, both in male and female AgAc-treated rats. The lower expression of symbiotic population of gut microbiome, as well as, an increase in the expression of bacterial genes representing *Enterobacteria* family may be a contributory factor for the gastrointestinal distress observed in AgAc gavaged rats.

Exposure to AgNP prompted size- and dose-dependent changes to ileal-mucosal microbial populations as well as of intestinal gene expression and induced an apparent shift in the gut microbiota towards greater proportions of Gram-negative bacteria. DNA-based analyses revealed that exposure to 10nm AgNP and low-dose silver acetate caused a decrease in populations of beneficial *Firmicutes* phyla, along with a decrease in the *Lactobacillus* genus.

The analysis of host gene expression in the ileum demonstrated that smaller sizes and lower doses of AgNP exposure prompted the decreased expression of important immune-modulatory genes,

including MUC3 (mucosal protective layer), TLR2 and TLR4 (involved in microbial recognition), GPR43 and FOXP3 (involved in T-cell regulation). Gender-specific effects of AgNP exposure were more prominent for the gut-associated immune responses. Although exposure to AgNP seemed to generally cause down-regulation of the examined genes, the AgAc and vehicle control animals seemed to show little change or a general up-regulation of gene expression. This seems to indicate that the gene expression responses observed in this study may be the result of nanoparticles-specific impacts and not simply the effect of silver ions. In any case, exposure to AgNP may lead to changes in the gut-associated immune response.

Summary

The presented data indicate that oral exposure of rats to silver acetate (64 mg Ag+/kg bw/d, lowest dose in the 90-day NTP study) caused alterations at the phylum-level in the ileum mucosa-associated microbiota and of the overall homeostasis of the intestinal tract. Oral exposure to Ag nanoparticles as well (lowest dose 9 mg AgNP/kg bw/d) prompted size- and dose-dependent changes to ileal-mucosal microbial populations, as well as, intestinal gene expression and induced an apparent shift in the gut microbiota towards greater proportions of Gram-negative *Enterobacteria* that may be the cause for the severe gastroenteritis. In addition, exposure to AgNP modulated the gut-associated immune response and the overall homeostasis of the intestinal tract.

Taken together, these changes in rat microbiome caused by exposure to silver ions or silver nanoparticles indicate that ingestion of silver affect the gastrointestinal tract function adversely.

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4.10.4 Summary and discussion of reproductive toxicity

The available data with respect to effects of reproductive toxicity/fertility can be discussed as follows:

- no statistically significant or clearly dose-related effects on fertility parameters were observed in the two-generation reproductive toxicity study with SZZ. Likewise, no effects on reproductive performance and reproductive organs were noted in the two-generation study with “silver containing active substance 2”.
- these findings appear to be consistent with results of studies on non-substituted zeolite A by Gloxhuber et al. (1983) and unpublished studies on synthetic zeolites reviewed by OECD (2006), which did not report any relevant signs of toxicity to reproductive organs. No effects on the testes of rats were observed after dietary exposure to about 1,250 mg/kg bw/d for 200 days. Therefore, the OECD set a NOAEL for male reproduction organs of about 1,250 mg/kg bw/d.

Taken together the findings on non-substituted zeolites as well as the silver-containing zeolites SZZ and “silver containing active substance 2”, it may be concluded that the modification of the zeolite

surface with silver atoms and their gradual release did not produce any substantial change in the reproductive effects of zeolites to rats.

Comparing the various adverse effects produced by dietary administration of both silver zinc zeolite substances to male and female rats it becomes obvious that reduced thymus weight in the offspring of both generations was caused by both SZZ and "silver containing active substance 2", i.e., at doses of approximately 510 mg/kg bw/d and 400 mg/kg bw/d or at Ag+ equivalent doses of about 11 or 12 mg/kg bw/d, respectively, and higher.

For comparative purposes, it is worthy of note that in an oral two-year chronic toxicity study with sodium aluminium silicate in Wistar rats, the relative weight of the thymus in females was affected significantly in the 100 and 1000 ppm groups (6.5 and 62 mg/kg bw/d, respectively; no further details reported) (Gloxhuber et al., 1983). In addition, pigmentation of glandular tissues/organs of both sexes was dose-dependently increased after administration of mid doses of both silver-containing zeolites (corresponding to about 11 mg Ag+/kg bw/d) and higher doses. However, no functional changes were observed.

With respect to toxicokinetics of sodium aluminium silicates in general, there is information that zeolites may partly hydrolyse during acidic conditions such as in the stomach, and their crystal structure is partly destroyed releasing silicic acid, as well as sodium and aluminium ions which could be taken up the gastrointestinal tract (Fruijtier-Pölloth 2009). After oral administration of synthetic zeolite A to rats, about 1% of the administered silicon was absorbed and eliminated via the kidneys and urine (Gloxhuber et al., 1983).

The major part of the administered zeolite A was excreted unchanged in the feces, thus, the intact molecule is not bioavailable after oral intake. In dogs, about 2-3% of silicon of the administered dose of zeolite A was absorbed by the gastrointestinal tract but there appeared to be no significant absorption of aluminium (Cefali et al., 1995, 1996, cf. Fruijtier-Pölloth 2009).

Sodium aluminium silicate did not cause any gross signs of adverse systemic effects in rats and mice after sub-chronic to chronic oral ingestion. Adverse effects in the kidneys and urinary bladder have been consistently reported, but the sub-chronic NOAEL for these effects in rats was in the range of 250-300 mg/kg bw/d (Gloxhuber et al., 1983). Adverse effects included deposition of crystalline material in the kidney and epithelial hyperplasia in kidney and bladder owing to mechanical damage due to the excretion of this material via the urine.

Overall, taking into consideration the kidney effects caused by zeolites it may be assumed that the dilation of the renal pelvis (hydronephrosis) observed on F1 animals in the two-generation study with SZZ may be due to zeolites per se, due to absorption of small amounts of silicon compounds from the gastro-intestinal tract after their partial hydrolysis to sodium, silicic acid and aluminium. Thus, the occurrence of hydronephrosis may be rather attributed to the zeolite structure than to a release of silver from silver zinc zeolite (SZZ).

4.10.5 Comparison with criteria

The CLP criteria relevant for developmental toxicity in the CLP guidance (2013) stated in section 3.7.2.2 (Table 3.7.1 (a)) can be summarised as follows:

- Category 1B Presumed human reproductive toxicant

"The classification of a substance in Category 1B is largely based on data from animal studies. Such data shall provide clear evidence of an adverse effect on sexual function and fertility or on development in the absence of other toxic effects, or if occurring together with other toxic effects the adverse effect on reproduction is considered not to be a secondary non-specific consequence of other toxic effects. However, when there is mechanistic information that raises doubt about the relevance of the effect for humans, classification in Category 2 may be more appropriate."

- Category 2 Suspected human reproductive toxicant

"Substances are classified in Category 2 for reproductive toxicity when there is some evidence from humans or experimental animals, possibly supplemented with other information, of an adverse effect on sexual function and fertility, or on development, and where the evidence is not sufficiently convincing to place the substance in Category 1. If deficiencies in the study make the quality of evidence less convincing, Category 2 could be the more appropriate classification.

Such effects shall have been observed in the absence of other toxic effects, or if occurring together with other toxic effects the adverse effect on reproduction is considered not to be a secondary non-specific consequence of the other toxic effects."

It is stated further (3.7.2.2.1.1) "In general all findings on reproductive toxicity should be considered for classification purposes irrespective of the level of parental toxicity. A comparison between the severity of effects on fertility/development and the severity of other toxicological findings must be performed."

With respect to developmental effects special emphasis is placed on maternal toxicity. The CLP guidance states in section 3.7.2.4 on maternal toxicity.

3.7.2.4.1. "Development of the offspring throughout gestation and during the early postnatal stages can be influenced by toxic effects in the mother either through non-specific mechanisms related to stress and the disruption of maternal homeostasis, or by specific maternally-mediated mechanisms. In the interpretation of the developmental outcome to decide classification for developmental effects it is important to consider the possible influence of maternal toxicity. This is a complex issue because of uncertainties surrounding the relationship between maternal toxicity and developmental outcome. Expert judgement and a weight of evidence approach, using all available studies, shall be used to determine the degree of influence that shall be attributed to maternal toxicity when interpreting the criteria for classification for developmental effects. The adverse effects in the embryo/fetus shall be first considered, and then maternal toxicity, along with any other factors which are likely to have influenced these effects, as weight of evidence, to help reach a conclusion about classification."

3.7.2.4.2. "Based on pragmatic observation, maternal toxicity may, depending on severity, influence development via non-specific secondary mechanisms, producing effects such as depressed fetal weight, retarded ossification, and possibly resorptions and certain malformations in some strains of certain species. However, the limited number of studies which have investigated the relationship between developmental effects and general maternal toxicity have failed to demonstrate a consistent, reproducible relationship across species. Developmental effects which occur even in the presence of maternal toxicity are considered to be evidence of developmental toxicity, unless it can be unequivocally demonstrated on a case-by-case basis that the developmental effects are secondary to maternal toxicity. Moreover, classification shall be considered where there is a significant toxic effect in the offspring, e.g. irreversible effects such as structural malformations, embryo/fetal lethality, significant post-natal functional deficiencies."

Comparison of the findings published by Shavlovski et al. (1995) with the classification criteria for developmental toxicity

Taken together, the available data on the impairment of copper status in rats and mice by silver allow the conclusion that the embryotoxic effects of silver chloride in rats following dietary administration of a high dose of 188 mg Ag/kg bw during the entire period of gestation (Shavlovski et al., 1995) can be considered not to be a direct effect of silver ions on the embryogenesis, but instead to represent a "secondary non-specific consequence" of the distinct disruption of maternal copper homeostasis in different tissues such as liver, kidney, heart, and placenta resulting in a severe copper deficiency.

In addition, the high silver levels occurring in circulation are in accordance with formation of Ag-modified ceruloplasmin containing 4 Ag atoms instead of Cu atoms with an irreversibly misfolded structure resulting in a functionally inactive ceruloplasmin which lacks enzymatic activity and copper transport function. Thereby, the availability of copper to the fetus is drastically reduced because plasma ceruloplasmin is the main source of copper for placenta and fetus. As a consequence of this, the development of the fetus and the pup viability are affected adversely.

In the same Shavlovski study, no developmental effects were found in the offspring of female rats treated only during the period of organogenesis with that high dose of 188 mg Ag/kg bw. Obviously, the shorter AgCl treatment did not yet result in systemic copper deficiency thus allowing undisturbed embryo-fetal development. In support of this finding it is to be noted that no maternal or developmental toxicity was produced in guideline-compliant oral prenatal development toxicity studies neither with "silver containing active substance 2" in rats (NOAEL 1000 mg/kg bw/d, Wood & Doleman, 1999) nor with the non-substituted Zeolite A in rats and rabbits with a NOAEL of 1600 mg/kg bw/d (cf. Fruijtier-Pölloth, 2009).

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In summary, the data on high dose administration of AgCl (188 mg Ag/kg bw/d) to female rats during the period of organogenesis did not reveal any developmental toxicity, while extended administration during the entire gestational period provided clear evidence for appearance of adverse effects on development, i.e. post-implantation deaths and pup mortality.

However, these adverse effects on development can be considered to be "secondary non-specific consequences" of the (i) disruption of maternal copper homeostasis in different tissues and (ii) the severe gastrointestinal disruption caused by a shift in microbial intestinal populations with an interference of essential micronutrient absorption, thus leading to copper deficiency in the offspring, which however does not constitute evidence for developmental toxicity of silver ions.

In this case, consequently, based on the available mechanistic information presented and the considerations given in the CLP guidance with respect to classification for developmental toxicity in the presence of maternal toxicity through "secondary non-specific mechanisms" related to the disruption of maternal homeostasis, it is not considered justified to use the Shavlovski (1995, on silver chloride) and Price (2002, on silver acetate) data on inorganic silver substances in support of classification of silver substances for developmental toxicity Category 1B or Category 2.

4.10.6 Conclusions on classification and labelling

The CLH proposal makes a statement in this chapter that "The mortality, the reduced pup weights and the reduced thymus weight observed cannot be explained by any unspecific effects in the mother".

This argument is clearly disputed, since there is ample information demonstrating that high oral silver administration causes severe gastrointestinal disruption in the mother animals by a shift in the intestinal microflora with subsequent homeostatic impairment. In addition, there is a homeostatic interaction of silver with copper homeostasis, whereby copper deficiency is induced, constituting a non-specific effect.

We politely note that there are many similar metal-metal interactions, which in the past have been discussed and dismissed for classification purposes. For the sake of brevity, the EU RAR of zinc and zinc compounds is cited here:

At high oral doses, zinc has been shown in developmental toxicity studies to induce maternal and fetal copper (and iron) deficiency. The EU RAR of 2004 concludes as follows: "Available data in animals on zinc excess indicate that adverse effects on fertility and foetal development may occur at dose levels of 200 mg Zn^{2+}/kg bw/day, in conjunction with other effects such as perturbation of parental and foetal copper homeostasis" and "As the margin between the dose at which in humans clinical signs are manifest and the dose at which in animals reproductive effects have been reported is so high (i.e. 80), it is considered unlikely that in humans reproductive effects will occur at exposure levels at which clinical signs are not manifest. Therefore, neither fertility nor developmental toxicity are considered end points of concern for humans. Based on the available information there is no reason to classify metallic zinc nor any of the zinc compounds considered for reproductive toxicity.

It is also worthy of note that any classification of zinc for reproduction toxicity would have had tragic consequences on nutritional and medical applications of zinc, since it has been shown that zinc supplementation at moderate levels in fact improves carriage and outcome of pregnancy and the health status of the newborn.

Reference: EU RAR (2004): Zinc Metal and Zinc Substances, Rapporteur: NL

4.11 Other effects

No comments.

5 ENVIRONMENTAL HAZARD ASSESSMENT

No comments.

6 OTHER INFORMATION

For purposes of comparison, reference is made above to human health data on "pure" (i.e., non-silver substituted) non-fibrous synthetic zeolites, for which the following summary is provided.

Introduction

Zeolites is a generic term applied to various crystalline aluminum silicates (Thomas & Ballantyne, 1992). There are both natural occurring and synthetic zeolites. The crystalline aluminum silicates have three-dimensional structures which contain cavities (i.e., micro-pores or interconnecting lattices) that can contain cations such as Na, K, Ca, Mg and may be filled with molecules of water. They possess excellent absorptive properties and also exhibit ion-exchange properties. More recently, synthetic zeolites have been produced for a variety of specialized applications as adsorbents, molecular sieves, ion exchangers and catalysts (Newsam 1986).

Synthetic zeolites designated by an arbitrary nomenclature according to their discoverers are mostly homocationic, i.e. they possess one cation species. The members of the synthetic, non-fibrous crystalline zeolites (zeolite A, zeolite P, zeolite X, zeolite Y) have an almost identical chemical composition, but the individual types have different crystalline structures resulting in type-specific binding characteristics. The so-called zeolite A is a synthetic sodium aluminum silicate with the formula:

$$Na_{12}(AlO_2)_{12}(SiO_2)_{12} \times 27\ H_2O$$

The toxicological data on zeolites have been summarized by Thomas & Ballantyne (1992) and more recently by industry (HERA 2004). Toxicity of zeolite nanoparticles which has been reviewed by Petushkov et al. (2010) emphasizing dependence on a number of parameters such as particle size, porosity, shape, surface area, surface functionalization, and surface treatment, is not subject to this survey.

Special attention was given to adverse effects resulting from long-term oral or inhalation exposure of experimental animals to zeolites. Data of this survey will be taken as a basis for examinations whether effects seen in studies with silver-containing zeolites can be attributed to the primary basic zeolite structure.

(1) Absorption, distribution, metabolism and excretion

At pH values below 4.0, such as in the stomach, zeolites may partly hydrolyze and their crystal structure is partly destroyed releasing sodium ions, silicic acid and aluminum which could be taken up the gastrointestinal tract (Fruijtier-Pölloth 2009).

Oral exposure of rats with up to 1000 mg/kg bw of various silicon-containing chemicals (zeolite A, sodium aluminum silicate, sodium silicate or magnesium tri-silicate) resulted in urinary excretion of silicon above background levels. The treatment with zeolite A did not result in an increased urinary excretion of aluminum (Benke & Osborne, 1979, cf. Fruijtier-Pölloth 2009).

After oral administration of 1000 mg/kg bw of zeolite A to rats, about 1% of the administered silicon was absorbed and eliminated via the kidneys and urine (Gloxhuber et al., 1983). The major part of the administered zeolite A was excreted unchanged in the feces. In the 90-day feeding study bladder calculi occurred in male rats fed 10000 ppm zeolite A via diet (Gloxhuber et al., 1983). Taken together, these findings proved that zeolite A was absorbed to a small extent after digestion. The absorption was presumed to take place only after breakdown of the zeolite A molecule since the silicon component but not aluminum could be traced in the urine.

Treatment of rats with 1000 ppm zeolite A (about 60 mg/kg bw/d) for 104 weeks resulted in a slightly, statistically non-significantly increased urinary excretion both of silicon and aluminum (Gloxhuber et al., 1983).

In dogs, about 2-3% of silicon of the administered dose of zeolite A (30 mg/kg bw) was absorbed by the gastrointestinal tract (Cefali et al., 1995, 1996, cf. Fruijtier-Pölloth 2009).

Synthetic zeolites are capable of exchanging their sodium ions with calcium and magnesium ions and with other cations, including trace elements (Kerr, 1989, Newsam, 1986, cf. Fruijtier-Pölloth 2009. It is therefore possible, that the bioavailability of some elements, such as e.g. Mg, Zn, and Cu might be influenced by zeolites. The cation exchange capacity (CEC) expressed in terms of milli-equivalents per gram is given as 5.48 meq/g for zeolite A. Based on this CEC, 1 g zeolite could bind 0.126 g Na^+, 0.214 g K^+, 0.067 g Mg^{2+}, 0.11 g Ca^{2+}, or 0.174 g Cu^{2+} (EFSA 2004).

(2) Acute toxicity

Rats tolerated a single oral dose of 10 g/kg bw of zeolite A without any overt reaction. Oral LD_{50} values > 16000 mg/kg bw were reported for rats and rabbits. Dermal LD_{50} values were determined to be > 2000 mg/kg bw for zeolites A and X (silver-containing). No toxic effects were observed after acute exposure to the zeolites A, Y and X, with 1-hour inhalation LC_{50} values of > 18300 mg/m^3 and > 2300 mg/m^3 for zeolites A and Y, respectively (Gloxhuber et al., 1983, Thomas & Ballantyne, 1992; OECD 2006).

(3) Irritation to skin and eyes

Zeolite A, Y and X were not irritating to the skin of rabbits, and slightly or moderately irritating to the rabbit eyes (OECD 2006). AZN zeolite (zeolite A substituted with Ag, Zn and NH3) was not irritating in a Draize test on the skin of New Zealand rabbits performed in accordance with OECD guidelines (Hirasawa et al., 1994b).

(4) Sensitisation

A skin sensitisation test with zeolite A in guinea pigs demonstrated the absence of a sensitizing potential (Gloxhuber et al., 1983). There is no evidence from human experience that zeolites may induce respiratory sensitization (OECD 2006).

(5) Genotoxicity

Zeolites A and X (silver exchanged) induced no gene mutations in several guideline tests on bacteria and mammalian cells in culture (only zeolite X was tested in the latter). Zeolite A was not clastogenic *in vitro*. *In vivo* clastogenicity studies considered to be reliable showed no evidence of induction of chromosomal aberrations by either zeolite A or X. However, a reliable *in vitro* study of zeolite X showed clear induction of chromosomal aberrations. While the difference in response between *in vitro* and *in vivo* is of interest, the important conclusion is that this category of chemicals lacks the potential to induce chromosomal aberrations in *vivo* (OECD 2006).

(6) Repeated dose toxicity and carcinogenicity

Oral route

In a 90-day feeding study SPF Wistar rats (20 animals/dose/sex) were administered to 0, 1000, 5000 and 10000 ppm zeolite A via diet (Gloxhuber et al., 1983). At the highest dose diminished urine secretion, hematuria and formation of ketone bodies was found. In 12/20 male rats urinary calculi were observed in the bladder, as well as a thickening of the wall. The histological examination showed a hyperplastic reaction of the transitional epithelium of the bladder in rats with calculi. In male animals without calculi, in the females of the 10000 ppm group and in all other groups no hyperplasia of the bladder epithelium was found. Thus, the cause of these changes appeared to be the mechanical effect of the calculi. The silicon concentration of the kidneys was significantly higher than in the controls, especially in males. No significant differences between the experimental groups and the controls were found in the metal contents in blood (iron), liver (copper and cobalt) and kidneys (zinc, aluminum and copper). Based on the changes at the highest dose, 5000 ppm (or approximately 250-300 mg/kg bw/d) was set as NOAEL in this study.

Groups of COX-SD rats were fed a diet with 0, 1250 or 20000 ppm (corresponding to approx. 75 and 1000-1200 mg/kg bw/d, respectively) of zeolite A for 160 or 200 days (Procter & Gamble 1975, 1976 as reported in Fruijtier-Pölloth, 2009). A significant increase in the incidence of kidney and bladder stones was observed in the high dose group. Other than this, there was no evidence of an alteration of urine parameters or kidney function. In this study, the dose of about 75 mg/kg bw/d can be set as NOAEL.

In an oral chronic toxicity and carcinogenicity study male and female SPF Wistar rats were fed 0, 10, 100 and 1000 ppm of zeolite A (corresponding to 0.62/0.65, 6.10/6.53 or 58.5/62.2 mg/kg bw/d for males and females, respectively) in the diet for 104 weeks (50 animals/dose group/sex) (Gloxhuber et al., 1983). Satellite groups of 15 males and 15 females were used for initial and interim investigations. Body weights and mortality rates of the treated groups were not significantly different from the control group. No treatment-related signs of toxicity were observed and no indication of a chronic response in any of the evaluated parameters was noted. Urinary excretion both of silicon and aluminum was slightly higher in the 1000 ppm group, but the difference was not statistically significant. The content of silicon was increased in the kidneys of males, but no significant differences were found in the contents of copper and cobalt in liver and of zinc and aluminum in kidneys. The organ weights of male animals showed no significant differences when compared to the control values. In the females, the relative weights of the adrenal glands of the 10 ppm group as well as of the thymus of the 100 and 1000 ppm groups differed significantly (no details reported). In animals that had died during the study or were sacrificed because of their poor condition, the main causes of death or ill health were basophilic adenoma and adenocarcinoma of the pituitary gland, adenoma and fibroadenoma of the mammary glands, subcutaneous fibroma and some tumors of the genital tract. No significant incidence of a particular type of tumor or of spontaneous mortality was evident in any group. No treatment-related findings were seen in any of the organs examined histologically, and there was no indication of any treatment-related induction of neoplasms. Accordingly, a NOAEL of 62 mg/kg bw/d can be derived from this long-term study.

The toxicity of a silver-containing zeolite (Zeomic, produced by Shinagawa Fuel Co.) was studied at dietary concentrations of 1.25, 2.5 and 5.0% in a 3-month oral study in Fischer-344 rats and mice (Hirasawa et al., 1994a). Pigmentations of pancreas, liver and kidneys were seen in all groups of treated-rats. Growth inhibition and mid-way death were observed in the 5% Zeomic group. In addition, a change in the vascular walls was reported presumably caused by deposition of silver. In mice, growth inhibition, atrial thrombus and death within 7 weeks were observed in the 5% Zeomic group. In the 1.25 and 2.5% groups, a dose-dependent pigmentation occurred. An increase of leukocytes and changes of other blood parameters (no data) were observed at 5.0%. According to the authors, the so-called non-effective dose of Zeomic for rats and mice was estimated to be 0.125%. However, this value might be a typing error because 1.25% was the lowest dose tested.

Zeolite A has the potential to reduce the risk of milk fever in dairy cows (EFSA 2004, 2007). It gradually prevents the decrease in serum calcium occurring after calving. The recommended zeolite A dose is 500 g/cow/day for two weeks prior to calving. Such zeolite A treatment did not affect serum levels of copper and zinc as well as milk composition, but induced a transient hypophosphatemia. The zeolite treatment of dry cows did not show any adverse effects in calves.

Conclusions: Sodium aluminum silicate did not cause any gross signs of adverse systemic effects in rats and mice after oral ingestion. Adverse effects in the kidneys and urinary bladder have been consistently reported in the repeated dose studies. The deposition of crystalline material in the kidney and the excretion of this material via the urine may cause mechanical damage in the kidney and bladder associated with concurrent epithelial hyperplasia in these organs. The NOAEL for these effects in rats was determined as 75 mg/kg bw/d in a 200-day study. No adverse effects were found in a 2-year study with rats at the highest dose tested (62 mg/kg bw/d).

In a Japanese oral subchronic study with administration of a silver-containing zeolite (Zeomic) to rats and mice growth inhibition and early deaths were reported at the highest dose of 5.0%

Inhalation route

Inhalation exposure of rats to zeolite A at a concentration of 20 mg/m^3 for 5h/d over a period of 13 days resulted in deposition of the substance in the lung parenchyma (Gloxhuber et al., 1983).

In a subchronic inhalation study 30 male rats and 5 guinea pigs were exposed to a mean zeolite A dust concentration of 2000 mg/m^3 for 5h/d on 5d/week over a period of 11 weeks (Gloxhuber et al., 1983). Three rats died during the test period at weeks 5, 7 and 10. All groups were affected by a respiratory infection. All animals showed signs of pneumonitis at both the gross and histological examination, but there was no indication of any fibrotic reaction. Silicon determinations on the lungs showed higher concentrations in the treated animals.

In a 90-day GLP-compliant inhalation toxicity study male and female rats were exposed nose-only to non-fibrous zeolite aerosol concentrations of 0.45, 1.9 and 10 mg/m^3 for 6h/d, 5d/week for 3 months (UOP LLC, 2002). The test substance was not further specified. For comparison, Quartz DQ12 (7.2 mg/m^3) and titanium dioxide (6.3 mg/m^3) were included as positive and negative control, respectively. Taking into account the different mass median aerodynamic diameters (MMAD) of the three test compounds, the aerosol concentrations of DQ12 and TiO_2 were adjusted with the 10 mg/m^3 zeolite group as basis to achieve the desired same retained lung burdens in the high dose groups. Upon cessation of exposure, a 3-month post-treatment observation followed. Effects indicating systemic toxicity were not observed in all groups. Clinical chemistry data did not reveal substance-related effects except significantly increased neutrophil and decreased lymphocyte levels in the zeolite high (females only) and DQ12 groups indicating a local pulmonary inflammation.

The desired equal lung retention of the test substances in the three high dose groups were achieved (about 1100 µg/rat in males and 1000 µg/rat in females). Overall, after the 3-month recovery period an actual retardation of lung clearance was barely seen, with the exception of the DQ12 group where clearance totally collapsed. Bronchoalveolar lavage showed a severe, persistent inflammatory response in the DQ12 animals. The analysis revealed a concentration-dependent increase in levels of polymorphonuclear neutrophils in the zeolite groups reaching the DQ12 level. These levels did not recover after the post-observation period. However, the response in DQ12 rats was 15-fold higher than in the high dose zeolite group.

Histopathological examination showed statistically significant exposure-related findings only in the lungs and lung-associated lymph nodes. There was no evidence of a systemic effect of the test substances used. Pulmonary inflammation was most pronounced in the quartz DQ12 group, followed by the 10 mg/m^3, 1.9 mg/m^3 and 0.45 mg/m^3 zeolite groups and the TiO_2 group. The inflammation in the quartz group was also qualitatively different as compared to the zeolite groups. Findings such as alveolar lipoproteinosis, formation of cholesterol granulas and the “mixed-type” of bronchiolo-alveolar hyperplasia were unique for the quartz group. Taken together, the NOAEL was $\leq$ 0.45 mg/m^3 zeolite in this 3-month nose-only inhalation study in rats.

Male and female Syrian hamsters were exposed to 0 and 20 mg/m^3 zeolite A for 5h/day, three days a week over a period of 12 months (Gloxhuber et al., 1983). The hamster study was terminated after 12 months because of deaths due to a specific infection. In the same study, groups of 15 male and 15 female Wistar rats were also exposed to 20 mg/m^3 zeolite A for 5h/day, three days a week for 22 months. The test material consisted of particles ranging from 0.5 to 10 µm, with most being less than 5 µm. Both species showed moderate to extensive signs of respiratory disease were seen in the treated and control animals. In the treated hamsters, macrophages containing accumulations of foreign material were found, mainly in the alveoli, but no signs of inflammation or connective tissue reaction were seen in the interstitial or alveolar region. In the rat lungs, greyish-white deposits were seen in the phagocytes of the alveoli or the peribronchiolar or perivascular areas as well as in the peribronchiolar lymph nodes near the hilus. Isolated deposits were seen also in the

mediastinal lymph nodes. No connective tissue reaction or other reactions were observed around these deposits. No tumors of the respiratory tract were diagnosed, but age-related tumors, especially adenomas of the pituitary gland, were found.

Cynomolgus monkeys (groups of 3 males and 3 females each) were exposed to zeolite A at concentrations of 0, 1, 6 and 50 mg/m^3 for 6h/day, 5 days a week for periods of 6, 12 or 24 months (Procter & Gamble, 1976, 1977, as reported in Fruijtier-Pölloth, 2009). Groups mean values for the MMADs were between 2.8 and 3.8 µm, indicating that a fraction of the generated dust has reached the alveolar regions of the lungs. There were no substance-induced histomorphological changes seen neither in the upper airways nor in any of the non-respiratory tract organs examined. The histopathological effects observed in a concentration-dependent manner in the lungs of animals of all groups were macrophage accumulations accompanied by sporadic bronchiolitis and alveolitis. No evidence of progressive pulmonary fibrosis was observed. The concentration of 1 mg/m^3 was considered as LOAEL.

Conclusions: Following 3-month exposure deposition of the test substance zeolite analog in lung was achieved. After the 3-month recovery period an actual retardation of lung clearance was barely seen. Available long-term inhalation studies with zeolite A in rats, hamsters, and monkeys gave no evidence of systemic toxicity, fibrosis or an increased tumor formation. For the lung effects, a LOAEL of 1 mg/m^3 for inflammation reactions was determined in long-term exposure of monkeys.

Dermal route

No data available.

(7) Reproductive and developmental toxicity

There were no fertility studies available. No signs of toxicity to reproductive organs by synthetic zeolites were reported in the unpublished studies reviewed by OECD (2006) or in the studies by Gloxhuber et al. (1983).

In 6-, 12-, and 24-month inhalation studies with cynomolgus monkeys, zeolite A has not shown any adverse effects on testes and ovaries up to and including a concentration of 50 mg/m^3. No treatment-related effects on the testes of rats were observed after exposure to up to 2.0% zeolite A in the diet (corresponding to about 1250 mg/kg bw/d) for up to 200 days. Therefore, the NOAEL for male reproduction organs was determined to be 1250 mg/kg bw/d (OECD 2006).

The teratogenic potential of a type A zeolite (Arogen 2000) was tested in Sprague-Dawley rats and New Zealand rabbits (Nolen & Dierkman, 1983, as reported in Fruijtier-Pölloth, 2009). Rats were treated at concentrations of 74 or 1600 mg/kg bw/d on gd 6-15 by gavage. Rabbits were given doses of 74, 345 and 1600 mg/kg bw/d by gavage on gd 6-18. The type A zeolite did not produce maternal or developmental toxicity in either rats or rabbits at the doses tested.

In a poorly documented Ukrainian study no effect on embryo-lethality and teratogenicity was reported on administration of 500 mg/kg bw of zeolite (not further specified) to an avian species (Bondarev et al., 2003).

Conclusions: There were no fertility studies available. No signs of toxicity to reproductive organs by synthetic zeolites were found in the studies reviewed by OECD (2006). Zeolite A did not show developmental toxicity in rat and rabbit studies performed similarly to OECD guideline 414, with a

NOAEL of 1600 mg/kg bw/d (highest tested dose) both for maternal toxicity and for developmental effects.

(8) Summary

After oral administration of zeolite A to rats, the major part was excreted unchanged in the feces. A smaller part was hydrolysed in the digestive tract and a silicon compound was absorbed and excreted via urine.

Sodium aluminum silicates showed a very low or no acute toxicity after oral or dermal administration or inhalation exposure. They were not irritating to the skin and slightly or moderately irritating to the eyes. Zeolite A demonstrated no sensitizing potential.

Zeolites A and X induced no gene mutations in bacteria and mammalian cells. Zeolite A was not clastogenic in vitro and in vivo.

Sodium aluminum silicates did not cause any gross signs of adverse systemic effects after repeated oral ingestion in rats. At high doses, adverse effects in the kidneys and urinary bladder have been consistently reported in the repeated dose studies. The deposition of crystalline material in the kidney and the excretion of this material via the urine may cause mechanical damage in the kidney and bladder associated with concurrent epithelial hyperplasia in these organs. The NOAEL for these effects in rats was determined as 75 mg/kg bw/d in a 200-day study. In a 2-year study in rats, there was no indication of any treatment-related induction of neoplasms or other adverse effects at the highest dietary level (60 mg/kg bw/d). A silver-containing zeolite (Zeomic) administered to rats and mice at a dose of 5.0% caused growth inhibition and early deaths as reported in an abstract of a Japanese oral subchronic study.

Available long-term inhalation studies with zeolite A in rats, hamsters, and monkeys gave no evidence of systemic toxicity, fibrosis or an increased tumor formation. For the lung effects, a LOAEL of 1 mg/m3 for inflammation reactions was determined in long-term exposure of monkeys.

There is no evidence that commercial synthetic zeolites can provoke significant pulmonary change since they are mainly non-fibrous. Conversely, fibrous natural zeolites (e.g., erionite) can produce pulmonary lesions (Thomas & Ballantyne, 1992).

There were no fertility studies available. No signs of toxicity to reproductive organs by synthetic zeolites were found in the studies reviewed by OECD (2006).

Zeolite A did not show developmental toxicity in rat and rabbit studies performed similarly to OECD guideline 414, with a NOAEL of 1600 mg/kg bw/d both for maternal toxicity and for developmental effects.

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