Harmonised Classification Proposal for Silver Nitrate

Comments from the European Biocidal Silver Task Force*

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Summary

DS Proposal	Hazard Code	STF Proposal	Hazard Code	Justification
Repr. 1B	H360FD	Repr. 2	H361FD	No substance specific data Uncertainty - read across is incomplete as whole substance effects (H314) are not addressed
Acute Tox 2	H300	No classification	-	Insufficient data
Skin Sens. 1	H317	No classification	-	Observed effects do not trigger classification
Muta. 2	H341	No classification	-	Insufficient data
STOT RE 2	H373	No comment	-	-
Ox. Sol. 1	H271	No comment	-	-
Skin Corr. 1A	H314	No comment	-	-
Aquatic Acute 1	H400	No comment	-	-
Aquatic Chronic 1	H410	No comment	-	-

Comments

Section 5 – Identified Uses

In the categories shown where silver nitrate is used directly as an active substance the concentrations used are very low. In consumer applications, where silver nitrate is typically incorporated into an inert matrix (polymer) to create treated articles, the amounts used are similarly low and exposure is further restricted only to silver (ions) migrating from the treated surface. The hazard thresholds discussed in this CLH proposal are many orders of magnitude higher than users will be exposed to in practice, particularly for consumers (general public). However, substance classification is only concerned with the hazardous properties of the substance itself, so reference to the use of silver nitrate is only for information and will not in fact contribute to the classification decision.

Section 6 – Data Sources

As described in this CLH Report and the RAC opinions for silver metal and silver zinc zeolite, it is generally accepted that toxicity can arise from exposure to the silver ion. Accordingly data available from studies conducted on a range of silver substances has been used collectively, employing weight of evidence or read-across principles to those data that are most relevant to the substance in question. On this point the RAC opinion for silver metal concluded '*In the absence of conclusive data on the substance itself, the classification of silver should ideally rely on extrapolations of toxicological properties from surrogate substances with sufficiently similar bioavailability, physical, chemical and toxicological properties*'.

It is important therefore to recognise that silver nitrate is a corrosive substance and as such has **toxicological properties that are significantly different to other silver salts and silver containing substances**. Due regard should be given to the effects of the counter ion (nitrate) and whether these effects have been given proper weight when reading across to the available data. Basing conclusions only on effects attributed to the silver ion in studies conducted with different silver salts will classify the 'silver ion', by itself this is not sufficient to classify silver nitrate without significant uncertainty.

Section 9.1 – Dermal Toxicokinetics

The ECHA guidance (ECHA, 2017) on dermal absorption makes specific reference to silver with respect to retention in the stratum corneum stating that the 'uptake of certain metal ions, 'particularly: Ag+ […] can be slowed as a result of binding to skin components'. Hostynek (2003) describes the process of binding to skin components as interaction with dermal proteins, which form a key part of the structure of the skin. In the case of silver these interactions may be seen as a visible skin discolouration (argyria) caused by the readiness of silver ions (Ag+) to be reduced to the metallic (Ag^0) oxidation state. Hostynek further describes the importance of metal binding in forming an active barrier in the skin that slows absorption and limits systemic circulation. As metal ions are retained in the upper strata of the skin further diffusion to lower layers is restricted due to the counter current created by the continuous natural upwards turnover of epidermal cells. With reference to cationic substances in general the ECHA guidance describes dermal absorption as very low, giving <1% as the range. Dissolved silver, or silver otherwise released from the SCAS will be present as the Ag+ cation and on this basis, silver may be regarded as intrinsically having a very low dermal absorption potential. The dermal absorption potential of silver was considered in a review of a silver based material by the Scientific Committee on Consumer Safety (SCCS) in 2016. The review was carried out under the scope of the Cosmetics Regulation (EC) No 1223/2009. The scientific data used by the SCCS comprised the work by Skog and Wahlberg (1964); a view by the US EPA (2011), discussed below; clinical studies of wound dressings and studies measuring the penetration of silver nanoparticles including the work by Larese et al. (2009).

Using these data, the SCCS made the following observations:

"*As an inorganic metal ion, the dermal absorption of silver is predicted to be very low based on its physicochemical properties (lipid solubility and molecular weight) and interaction with dermal proteins (Hostynek, 2003). This prediction is confirmed by observations of Skog & Wahlberg (1964) who report the dermal absorption of silver nitrate through intact guinea pig skin to be less than 1% after 5 hours of exposure (based on the limits of detection), and Snyder (1975), who reports a similar finding for human skin. Clinical and experimental studies indicate that the dermal absorption of silver is 'exceedingly low' (Lansdown, 2010); this observation is attributable to the barrier properties of epidermal phospholipids and the irreversible binding of free silver ions to keratin sulphydryl groups. More recent investigations of the dermal absorption of silver from impregnated nanosilver dressings in burns patients report a level of dermal absorption of 0.1% or less, even in severely damaged skin [US EPA assessment of data reported by Moiemen et al., 2011; US EPA slides 19 and 20]. The authors indicate that this level of absorption is orders of magnitude higher than that measured in intact skin.*"

In conclusion, the SCCS made the following comment:

"*The level of 0.1% dermal absorption is considered to be relevant … and is used for calculations of systemic exposure to silver resulting from the use of this product as cosmetic packaging material. The value of 0.1% for dermal absorption of silver is derived from serum levels in persons with severely damaged skin (burn patients) during treatment with silver-rich wound dressings (Moiemen et al., 2011; US EPA 2011). This value is a conservative upper-bound estimate and orders of magnitude higher than those measured in vitro with intact or mildly damaged human skin (Larese et al. 2009).*"

In concluding a percentage dermal absorption value of 0.1% the EPA used the Moiemen et al (2011) study, which assessed plasma silver levels in burn patients following the use of wound dressings containing silver; in combination with the in vitro studies by Larese (2009). This was a weight-of-the-evidence approach to quantitatively estimate dermal penetration of silver from nanosilver applied to treated articles. This opinion by the US EPA was considered to be a conservative (worst-case) estimate since the

patients in the study had damaged skin and the method of calculation assumed that all circulating serum silver resulted from the dermal absorption of silver from the wound dressing, instead of innate silver that may be present, or that silver deposited in the skin layers may be due to impaired clearance in the damaged skin.

Based on weight of evidence from a number of published papers and the opinion of regulators a dermal absorption value of 0.1% is appropriate for systemic exposure to silver following dermal contact.

Published References

ECHA (2017). Guidance on the Biocidal Products Regulation Volume III Human Health - Assessment & Evaluation (Parts B+C) Version 4.0 December 2017.

EPA (1989). Integrated Risk Information System (IRIS). Silver (CASRN 7440-22-4). http://www.epa.gov/iris/subst/0099.htm

Hostynek, J.J., (2003). Factors determining percutaneous metal absorption. *Food and Chemical Toxicology*. **41**, 327-345.

Lansdown, A.,B., G., (2010) A pharmacological and toxicological profile of silver as an antimicrobial agent in medical devices. *Adv Pharmacol Sci*, Article ID 910686, 16 pages; doi:10.1155/2010/910686

Larese FF, D'Agostin F, Crosera M, Adami G, Renzi N, Bovenzi M, Maina G (2009). Human skin penetration of silver nanoparticles through intact and damaged skin. *Toxicology* **255** (1-2):33–37.

Moiemen NS, Shale E, Drysdale KJ, Smith G, Wilson YT & Rapini R. (2011). Acticoat dressing and major burns: systemic silver absorption. *Burns* **37** (1), 27-35. doi: 10.1016/j.burns.2010.09.006

Skog, E.; Wahlberg, J.E. (1964). A comparative investigation of the percutaneous absorption of metal compounds in the guinea pig by means of the radioactive isotopes: ^{51}Cr, ^{58}Co, ^{65}Zn , ^{110m}Ag, ^{115m}Cd, ^{203}Hg. *J. Invest. Dermatol*. **43**:187-192.

Snyder W.S. (1975). Report of the Task Group on Reference Man. Pergamon Press, Oxford, England, pp. 407-708. (Cited in ATSDR, 1990).

Section 10.1 – Acute toxicity – Oral route

Silver nitrate is corrosive and robust acute toxicity data are lacking for animal welfare reasons due to the certainty of effects if dosed directly via the oral route. Repeating such studies would be unethical. Read across to data from other silver substances and silver salts is not justified because corrosive properties are unique to silver nitrate. Although data suggest classification with Acute Oral Category 2 (5 < ATE ≤ 50) is indicated, the data are of insufficient quality to conclude this level of classification with certainty and as such **no classification should be proposed for silver nitrate on the basis of insufficient data**.

Section 10.7 – Skin sensitisation

The data submitted to propose classification for silver nitrate for skin sensitisation (Category 1) is largely the same data that were submitted to propose classification for silver metal and silver zinc zeolite. The RAC opinions for these substances based on the available data concluded that there was no concern for skin sensitising potential for both substances.

- silver metal: '*RAC disagrees with the DS on the weight of the evidence, in particular on the interpretation of the results from the two Buehler studies. RAC considers that no classification for skin sensitization is warranted.*'

- silver zinc zeolite: '*In conclusion, RAC agrees with the DS that no classification of silver zinc zeolite for skin sensitisation is warranted*'.

Accordingly, and to be consistent with the CLH decision for silver metal and silver zinc zeolite, the classification recommendation for **silver nitrate** should be **no classification for skin sensitisation**.

Section 10.8 - Classification for germ cell mutation (proposed Category 2)

Overview

The data submitted to propose classification for **silver nitrate** for germ cell mutation (Category 2) is largely the same data that were submitted to propose classification for **silver** (CAS No. 7440-22-4). The new data submitted for silver nitrate are limited to 9 published articles describing in-vitro studies. These studies are old, poorly reported and not conducted to GLP or any test guideline. The results are negative for effects with bacteria (6 studies) which, as has been noted by RAC, are influenced by the bactericidal properties of silver and are therefore of limited value. The conclusions regarding positive effects in the 3 remaining studies are overstated given the incomplete nature of the data and the fact that the results of the in-vitro data with silver complexes (SCAS), to which they are linked by the DS, are not universally positive but instead show a mix of positive/negative and equivocal results. The mutagenicity data submitted in the CLH dossiers for both silver nitrate and silver consist of 13 studies conducted in-vitro with silver complexes (SCAS) and 6 studies conducted in-vivo with the same SCAS and 3 studies conducted in vivo with nanosilver.

Based on the RAC opinion for silver (CLH-O-0000007152-82-01/F, Page 46), the **in vitro dataset** for silver nitrate can be regarded in the same way as silver, being *"sparse and incomplete for ionic (soluble) salts and Ag+-releasing SCAS and they are more ideally considered to supplement the data for silver and be weighed on a case-by-case basis as such*".

Based on the RAC opinion for silver (CLH-O-0000007152-82-01/F, Page 52), the **in vivo dataset** for silver nitrate, although reliable, can in the same way as silver be considered lacking, because "*SCAS such as zeolites and other ion exchangers may not be relevant because they release only small amounts of silver ion that cannot be properly tested and are likely to give a negative result because insufficient silver ions are available to the testing environment*".

The overall conclusion for the classification of silver nitrate for mutagenicity can be based on the RAC opinion for silver (CLH-O-0000007152-82-01/F, Page 54) which states that "*while the mutagenicity database for silver is extensive for several forms and compounds of silver, the data are inconclusive overall because of contradictory findings and in many cases a lack of sufficient information for each study report. Some concerns remain with respect to the in vivo findings for both chromosomal aberrations and DNA strand breaks but the negative results generally in this case outweigh the positive ones*". Accordingly, and to be consistent with the CLH decision for silver, the classification recommendation for **silver nitrate** should be **no classification for mutagenicity due to inconclusive data**.

Comments on DS comparison with the CLP criteria

Section 10.8.2, Paragraph 1, Page 79.

There are no robust data to assess if silver nitrate has a potential to induce germ cell mutagenicity.

- The lack of substance specific data for silver nitrate is the same situation for the classification of silver. The conclusions in the RAC opinion for silver (CLH-O-0000007152-82-01/F, Page 53) are therefore applicable to the current classification proposal for silver nitrate. Notably the following:

- *There is no direct evidence in humans that elemental silver, or **ionic silver substances**, are able to induce heritable genetic mutations in humans (nor is there useful data on somatic cell mutagenicity in humans).*

- *The selection of studies presented in the CLH report are representative of what is publicly available.*

- *It is unlikely that the incorporation of more published studies would clarify the situation further.*

- *It is acknowledged that most of the published studies, irrespective of whether they are positive or negative, suffer from deficiencies compared to guideline compliant studies. In this case it is not feasible nor is it advisable to forensically analyse each and every publication. It is appropriate to judge whether a given study is acceptable or not and regardless of deficiencies, provide sufficient information on the genotoxic potential of silver, silver nanoparticles or silver ions.*

Section 10.8.2, Paragraph 1, Page 79.

Results obtained among existing in vitro studies with silver nitrate and various ionic silver substances in mammalian cell gene mutation tests, micronucleus assays and chromosomal aberration tests provide a range of negative and equivocal results for mutagenic activity as well as positive findings.

Most published studies were non-GLP and not performed according to any OECD test guideline but overall, the in vitro data indicates a concern for DNA damage / gene mutation and clastogenicity for silver nanoforms and other silver compounds including silver salts.

- Results from in vitro studies with SCAS (Table 46) are mixed. Equivocal/positive responses are reported in mammalian cell assays mainly without S9 and these responses are only observed at the threshold of cytotoxicity. In the classification opinion for SZZ (CLH-O-0000001412-86-90/F, Page 24) RAC noted that "*results obtained with and without S9 mix should theoretically yield the same qualitative, if not quantitative results. Although speculative, a possible explanation for the differences might be related to a potential complexation of silver ions with thiols and disulfide groups in the S9 mix, reducing its availability to the cells*".

- The CLH dossier contains no studies on the behaviour of AgNPs in test systems in vitro (Table 46), so these data should not be relied upon or referenced. The argument that the effects of AgNPs are due to solubilised silver ions is simplistic, particularly for in vitro systems.

- Some concern remains for soluble salts due to the equivocal/positive findings seen with SCAS. These conclusions are already reflected in the existing RAC opinion for silver (CLH-O-0000007152-82-01/F, Page 53) based on the same data. Notably the following: "t*he in vitro data indicates a concern for DNA damage / gene mutation and clastogenicity for silver nanoparticles and other silver complexes and salts requiring further verification with in vivo studies".*

Section 10.8.2, Paragraph 1, Page 79.

Unfortunately, there is no appropriate in vivo data on silver nitrate. However, data on nanosilver and silver acetate available in the open literature is considered to provide information on the genotoxic potential of silver ions and thus the properties of silver nitrate at non-corrosive concentrations.

Although data obtained at concentrations where corrosivity would determine the toxicity of silver nitrate and data would thus not be reliable for the assessment, it should be noted that since the threshold of corrosivity is not known, all data is currently considered in the weight of evidence approach used in this

CLH report. Based on the results of the 28-day study it can be concluded that at least systemic doses up to 100 mg/kg bw/d are well-tolerated by rats.

- Silver nitrate is presently classified for corrosive properties; Skin Corr. 1B according to CLP. According to the DS evaluation in Section 10.4.3 of the classification proposal, silver nitrate meets the criteria for a lower threshold and should be classified as Skin Corr. 1A. Developing in vivo mutagenicity data is therefore restricted because testing is unethical due to the corrosive properties of silver nitrate.

- According to the DS an exact LD50 value cannot be set, but the animal data generally referred to in different reviews indicate oral LD50 values between 50 and 1173 mg silver nitrate/kg bw. Toxicity is likely to be influenced by the route of administration, with gavage application (in the case of the referenced 28-day RDT study, IIIA 6.3.1-07) likely to by-pass the primary acute effects since silver nitrate will be rapidly converted to chloride or chloride complexes when introduced directly into gastric media. The effect of directly dosing silver nitrate via the oral route is therefore unknown but is most likely to be less well tolerated than is indicated by the 28 day gavage study IIIA 6.3.1-07.

Section 10.8.2, Paragraph 1, Page 79.

Data on nanoparticles submitted by the applicant for the review under BPR includes two bone marrow micronucleus studies with negative results. Exposure of the target tissue was not demonstrated by the PCE/NCE ratio, but systemic availability is indicated by data showing distribution to blood, stomach, brain, liver, kidneys, lungs and testes and is considered sufficient evidence for target tissue exposure. In contrast to these negative bone marrow micronucleus studies, the results from another study found in the open literature indicate that nanoparticles of silver induce irreversible chromosomal damage in the bone marrow and cause large DNA deletions in developing embryos of mice. The study was not performed according to the principles of GLP or any OECD test guideline but is published in a peer-reviewed scientific journal and the results are thus considered reliable. This data gives no clear picture of the intrinsic properties of the substance. `

- Reliance on data generated using AgNPs is not appropriate due to significant differences in kinetics and deposition between AgNPs and soluble silver salts. Furthermore, the inconsistent nature of the AgNP dataset indicates that the studies are either of questionable reliability or there is the likelihood that different AgNPs (size, shape, surface coating) give different responses.

Section 10.8.2, Paragraph 1, Page 79.

Whereas the two bone marrow micronucleus studies indicate a low concern for mutagenicity, the chromosomal damage in bone marrow and large DNA deletions in developing embryos of mice observed in a different study are alarming. Consequently, one study indicates that the second paragraph in CLP annex I: 3.5.2.2 is fulfilled whereas two others indicate the opposite. An unclear picture remains also when taking into account all data available for different silver compounds and different types of in vivo tests.

- Data are clear if the studies with AgNPs are discounted. There is no evidence for chromosomal effects, and insufficient data are remaining to conclude on gene mutation.

Section 10.8.2, Paragraph 2, Page 79/80.

Results for SCAS such as silver zeolites and other ion exchangers are considered less relevant since they release only small amounts of silver ions that are insufficient to be properly tested in these assays and will thus almost always give a negative response.

- The comet assay with SZZ (IIIA 6.6.5-02) was dosed at 2000 mg SZZ/kg bw, which is equivalent to *ca* 68 mg/kg bw silver, or *ca* 100 mg/kg bw silver nitrate. The study was negative. The comet assay was developed to give further information on the exposure of target tissues in the in vivo chromosome aberration assay conducted with SZZ (IIIA 6.6.4-01). The conclusions given in the Competent Authority Report for SZZ on these studies states the following:

  "In the dossier for silver zinc zeolite (SZZ) data investigating genotoxic potential in-vivo was submitted in the form of a rat chromosome aberration (CA) assay (IIIA 6.6.4-01) conducted with Hygentic B8000 as the test material. Although the assay gave negative results following oral dosing up to 5000 mg/kg bw the evidence of test article exposure to the target tissue (bone marrow) was considered to be inconclusive. As a consequence, further data on genotoxicity was requested by TMII (June 2013) and an in vivo Comet assay was agreed the most appropriate test.

  As in-vivo genotoxicity data had been generated using Hygentic B8000 it was considered appropriate to validate the existing data by using the same test material in the Comet assay. In a pre-validation study exposure of the target (blood plasma) with silver was shown at doses of silver-zinc-zeolite that were used in the in vivo CA assay (IIIA 6.6.4-01). The choice of test material and results of the pre-validation study were communicated to the eCA in November 2015 and April 2016 in the form of the test protocol and a short report of the pre-validation results. The test protocol, in which a detailed description of the silver content of the test material was included, was received without comment and the eCA acknowledged that exposure of the target organ is sufficiently demonstrated".

- The rat chromosome aberration (CA) assay (IIIA 6.6.4-01) in conjunction with the Comet Assay (IIIA 6.6.5-02) confirm negative effects for genotoxicity. The dose applied in the CA assay was 5000 mg SZZ/kg bw which is equivalent to *ca* 175 mg silver/kg bw, or *ca* 275 mg silver nitrate/kg bw. The potential for genotoxic effects has therefore been tested at levels higher than those quoted by the DS. Whilst the levels tested do not achieve the required limit concentration (in the absence of any expected general toxic effects) in the guidance, they do at least indicate no significant concern for genotoxicity that could warrant classification.

Section 10.8.2, Paragraph 2, Page 80.

Overall, the most relevant in vivo study to represent silver nitrate is the micronucleus study performed with silver acetate as it is both robust and performed with a soluble salt with no expected impact of confounding factors.

- This study is the published research by Boudreau et al (2016). The RAC opinion for silver (CLH-O-0000007152-82-01/F, Page 54) concluded that "*Boudreau et al., 2016 presents valuable information with silver acetate, showing that the release of copious amounts of silver ion does not give rise to a positive in vivo genotoxic response*".

Section 10.8.2, Paragraph 2, Page 80.

Nevertheless, this type of study does not address the concerns for gene mutation indicated by in vitro tests and several in vivo comet assays.

- The negative comet assay with SZZ (IIIA 6.6.5-02) indicates a lack of concern at a relatively low dose level (68 mg/kg bw silver), although this dose level is within the range of positive results reported for oral studies with AgNPs (further casting doubt on the relevance of the AgNP data). Removing the questionable reliance on AgNP data results in a lack of robust information to address this endpoint.

Section 10.8.2, Last Paragraph, Page 80.

Based on the results obtained with nanosilver releasing silver ions, the criteria for classification are considered fulfilled.

- It is not appropriate to rely on AgNP data to support classification of silver compounds due to significant differences in kinetics and deposition between AgNPs and soluble silver salts. Furthermore, the inconsistent nature of the AgNP dataset indicates that the studies are of questionable reliability and there is the likelihood that different AgNPs give different responses

Section 10.8.2, Last Paragraph, Page 80.

The data is difficult to interpret but the overall weight of evidence is considered to indicate that silver ions do not induce micronuclei but may induce gene mutations.

- The weight of evidence is insufficient to conclude on gene mutation.

Section 10.8.2, Last Paragraph, Page 80.

Silver is detected in gonads and has been shown to cause adverse effects in germ cells.

- Silver is detected in many organs/tissues, often without any associated damage. While there is (inconsistent) evidence of testicular toxicity in published studies with AgNPs, there is little evidence of testicular toxicity occurring in the more reliable studies with SCAS and soluble silver salts.

Section 10.8.2, Last Paragraph, Page 80.

However, considering the variations in data and the limitations of each individual study, data is not considered sufficiently robust to fulfil criteria for classification in category 1B and category 2 is thus considered appropriate. This is supported by the CLP guidance (page 366; ECHA, 2017) stating that: "A complex data situation with positive and negative results might still lead to classification. This is because all tests detecting a certain type of mutation (e.g. point mutations) have been positive and all tests detecting chromosome mutations have been negative. Such circumstances clearly warrant classification although several tests have been negative which is plausible in this case. Consequently, silver nitrate is considered to fulfil criteria for classification and labelling in category 2.

- Comet assays were performed using AgNPs and a variety of dosing routes, however it is difficult to justify using the results of AgNP studies using exposure routes other than oral. Two papers using oral dosing are cited as being reliable.

  Patlolla *et al*. (2015) used five consecutive oral doses at levels of up to 100 mg/kg bw/d. They report the induction of oxidative damage, cytotoxicity (reduced mitotic index), clastogenicity and DNA damage (comet formation). DNA damage was significantly increased by treatment with 50 and 100 mg/kg bw/d.

  Awasthi *et al*. (2015) used oral dosing of AgNPs in mice and report DNA damage in the liver following single dose levels of 50 and 100 mg/kg bw, and repeated (weekly) dosing with 10 and 20 mg/kg bw. Findings were associated with hepatotoxicity, demonstrated by elevations in serum enzyme activities and histopathological change.

  Compare these with the 28-day oral study with silver nitrate in rats, which does not show liver damage at dose levels of up to 100 mg/kg bw/d. While Boudreau et al (2016) shows the deposition of silver particles following oral dosing with AgNPs and a silver salt (silver acetate), they noted clear

differences in morphology and distribution. Following dosing with AgNPs silver deposits were predominantly intracellular, whereas deposits following dosing with silver acetate were associated extracellular membranes.

These findings therefore bring into question the reliance on data generated using oral dosing with AgNPs. It is notable that the DS cites a published paper looking at the responses to AgNPs and silver sulphate (Sycheva *et al*., 2016) noting differences in responses but is discounted this because the results are 'inconsistent', rather than casting doubt on the AgNP/silver salt equivalence argument.

Data with AgNPs are inappropriate, this leaves insufficient data to address the potential for gene mutation in vivo with silver nitrate and the recommendation for silver nitrate should therefore be no classification for mutagenicity due to inconclusive data.

Published References

Awasthi KK, Verma R, Awasthi A, Awasthi K, Soni I, John PJ (2015) In vivo genotoxic assessment of silver nanoparticles in liver cells of Swiss albino mice using comet assay. Adv Mater Lett. 6: 187–193.

Boudreau MD, Imam MS, Paredes AM, Bryant MS, Cunningham CK, Felton RP, Jones MY, Davis KJ, Olson GR (2016) Differential effects of silver nanoparticles and silver ions on tissue accumulation, distribution, and toxicity in the Sprague Dawley rat following daily oral gavage administration for 13 weeks. Toxicol Sci. 150: 131-160.

Patlolla AK, Hackett D, Tchounwou PB (2015). Genotoxicity study of silver nanoparticles in bone marrow cells of Sprague-Dawley rats. Food Chem Toxicol. 85: 52-60.

Sycheva, L. P.; Murav'eva, L. V.; Zhurkov, V. S.; Mikhailova, R. I.; Savostikova, O. N.; Alekseeva, A. V.; Sheremet'eva, S. M. (2016) Study of cytogenetic and cytotoxic effects of nanosilver and silver sulfate in germ cells of mice in vivo. Nanotechnologies in Russia, 11(3-4), 256–262.

Section 10.10 - Reproductive toxicity

Overview

There are no substance specific data on the reproductive effects of silver nitrate. In the absence of data, existing studies conducted mostly with silver acetate have been used in a read-across approach by the DS in order to conclude classification for silver nitrate. The assumption in this approach is that the observed reproductive toxicity is derived from the silver ion.

According to the ECHA Guidance on Grouping of Chemicals (Chapter R.6) the effect of the counter ion should also be taken into account for read across between metal compounds. This is particularly important in the case of silver nitrate as there are fundamental toxicological differences between silver acetate and silver nitrate.

In various parts of the CLH dossier the bioavailability of silver (ion) from silver acetate and silver nitrate is described as being similar, with an implication that effects observed with silver acetate can therefore be read across directly to silver nitrate for classification. The evidence given for similarity is the comparative toxicokinetic study (Anonymous et al – 2021) submitted in Section 9. It is important to note that in this study silver acetate and silver nitrate were administered via gavage and therefore directly introduced into the gastric fluid. In this situation, silver acetate and silver nitrate will both be rapidly converted to the same chloride/chloride complexes, so in this case it is unsurprising that silver (ion) bioavailability is similar.

Data measuring the effects of dosing silver nitrate directly to test animals are lacking. The 28 day RDT study IIIA 6.3.1-07 is cited as indicating systemic doses up to 100 mg silver nitrate/kg bw/d are well-tolerated by rats, but this study has the same limitation created by gavage dosing as described above. Direct acute or corrosive/irritation effects are by-passed by gavage application, so it remains unknown whether the reproductive toxicity data developed using dietary administration of silver acetate are directly applicable to silver nitrate in a read-across framework. The study by Matuk (IIIA 6.5-03) contains some evidence of significant toxicity (rapid weight loss from week 23 onwards and eventually death) following administration at 222 mg/kg bw via drinking water, but without comparable silver acetate data the influence of the nitrate anion compared to the acetate anion is unknown.

According to the RAC opinion for silver (CLH-O-0000007152-82-01/F, Page 83), "*the data available on silver acetate and nanosilver indicate that **the silver ion** can cause adverse effects on sexual function and fertility possibly by a mechanism involving oxidative stress and perturbations of Cu homeostasis. However, a classification of 1B is dependent on having sufficiently robust data to support the proposal*".

In the case of silver nitrate, although the mechanism of toxicity via silver ion exposure is plausible, experimental data are lacking to determine the influence of nitrate on general toxicity (acute effects and local corrosivity) and whether or not this would exacerbate effects seen with silver acetate to the point where the animals would suffer more marked general toxicity that would preclude classification.

Substances are classified in Category 2 if 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."

Since evidence of the contribution to toxicity from the nitrate anion is absent this should present sufficient concern for uncertainty. Classifying silver nitrate in Category 1B based solely on extrapolating silver ion exposure from silver acetate should not be warranted for effects on sexual function and fertility, and effects on development. **The concerns linked to effects resulting from the silver ion are adequately addressed with Category 2 according to the above criteria, with classification at this level then consistent with the existing RAC opinions for metallic silver and silver zinc zeolite which are based largely on the same data supporting the current proposal**.

Section 10.10.1 - Effects on sexual function and fertility (proposed Category 1B)

Comments on DS comparison with the CLP criteria

Section 10.10.3, Paragraph 5, Page 155.

The reduction of the fertility index (10%, not statistically analysed) and the statistically significant reduction of the number of implantations (22%, 11.3 compared to 14.4 in control) in dams observed in the published study with silver acetate at 40 mg/kg bw/day (IIIA 6.8.2-06) are considered to represent "clear" evidence of an adverse effect on sexual function and fertility and thus to fulfil criteria for classification in category 1B.

- In study IIIA 6.8.2-06 (Sprando, 2016) the fertility parameters that were lower at the high dose level (40 mg/kg bw/d) were fertility index (90% compared to 100% in controls) and implantation numbers (11.3 compared to 14.4 in controls), which also resulted in a smaller litter size (10.3 compared to 13.1). However, these parameters were not affected by treatment in the EOGRTS with silver acetate (dose levels of up to 120 mg/kg bw/d) or in the range-finder for this study (dose levels of up to 320 mg/kg bw/d). Similar effects were also not seen the 2-generation studies with SZZ or SZZHP. The

findings reported in IIIA 6.8.2-06 therefore have to be considered in light of the entire dataset and by themselves do not support classification in Category 1B.

Section 10.10.3, Paragraph 5, Page 155.

Oestrus cycle, sperm parameters and histopathological analyses of reproductive tissues (other than the testis) were not investigated in the one-generation study with silver acetate thus it is not known if the reduced fertility index results from a toxicological effect on germ cells.

- There is sufficient evidence from other reliable studies with silver acetate (published 90-day study by Boudreau dosing up to 400 mg/kg bw/d), the EOGRTS RF study (up to 320 mg/kg bw/d) to rule out direct effects on germ cells. Germ cell toxicity is also not a feature of SCAS toxicity.

Section 10.10.3, Paragraph 5, Page 155.

However, the results from a recent EOGRTS study showed statistically significant decreases in cauda epididymis and testicular weight in F1 males administered 80 and 120 mg/kg/day and low testicular and cauda epididymal total spermatid and sperm counts (millions) at all dose levels. Minor effects were also observed in high and mid dose males and in F0.

- These findings in the EOGRTS are not very convincing in terms of a relationship to treatment, and certainly not of sufficient magnitude to influence male fertility. There is no effect on fertility in the EOGRTS at dose levels of up to 120 mg/kg bw/d, or in the one-generation RF study at dose levels of up to 320 mg/kg bw/d. There is no effect on sperm parameters in the Boudreau study (dose levels of up to 400 mg/kg bw/d). Consequently, there is no clear effect on fertility and no link between sperm effects and fertility that should require classification in Category 1B.

Section 10.10.3, Paragraph 5, Page 155.

The number of implantation sites (15.6, 16.9, 16.3 and 16.1 at 0, 40, 80 and 120 mg/kg bw/day, respectively) and fertility indices (100, 100, 96, 96% at 0, 40, 80 and 120 mg/kg bw/day, respectively) were not affected by treatment in the EOGRTS on silver acetate.

- The results of this study should carry more weight than the results of the Sprando (2016) study.

Section 10.10.3, Paragraph 6, Page 155.

Furthermore, the results from several studies performed with nanosilver support an effect of silver ions on germ cells as they show a reduced number of sperm and alterations in sperm morphology…

- We should not be relying on studies with AgNPs due to fundamental differences in toxicokinetics compared to soluble silver salts.

Section 10.10.3, Paragraph 6, Page 155.

The studies are not performed according to guidelines or the principles of GLP hence fewer animals and dose levels than required in guidelines were used in most of the studies. Therefore, it is difficult to assess the reliability and relevance of the results. However, since similar effects were observed in several studies and these are published in peer-reviewed scientific journals, the quality of studies is yet assumed to be acceptable.

- It is agreed that published studies with AgNPs are less reliable. We cannot simply accept the results of these studies just because there are a lot of them, particularly given notable inconsistencies and discrepancies in the reported results.

Section 10.10.3, Paragraph 6, Page 155.

The results indicate that nanoparticles of silver have acute and significant effects on spermatogenesis, the number of spermatogenic cells and on acrosome reaction in sperm cells.

- Published research frequently but not universally reports testicular toxicity and/or effects on sperm parameters in animals administered AgNPs by different exposure routes. Published studies report sperm effects in association with testicular toxicity, which is not characteristic of soluble silver salts, even at much higher dose levels. There is also a great deal of variability in the published data; some studies reporting toxicity at very low dose levels and others reporting no effects at much higher dose levels. Thakur *et al*. (2014) reported testicular toxicity in rats dosed orally with 5-20 nm AgNPs for 90 days at a dose level of 0.02 mg/kg bw/d. In contrast, in a study of the same duration, Boudreau *et al*. (2016) report an absence of testicular toxicity or effects on sperm parameters in rats dosed orally with 10, 75 or 110 nm AgNPs at dose levels of up to 36 mg/kg bw/d. Kim *et al*. (2010) also report an absence of effects on testicular histopathology in a 90-day rat study with 56 nm AgNPs at dose levels of up to 56 mg/kg bw/d.

Section 10.10.3, Paragraph 6, Page 155.

Effects on germ cells were also noted in the two-generation studies performed with silver sodium hydrogen zirconium phosphate and silver zinc zeolite but in the absence of statistical significance and effects on reproductive performance, the significance is difficult to assess.

- Not considered to be relevant; studies also considered by the DS to be of lower relevance due to the potential (unknown) impact of other components present in these SCAS.

Section 10.10.3, Paragraph 6, Page 155/156.

Moreover, a statistically significant delay in the onset of puberty was observed at both doses tested in one study with nanosilver although the weight at puberty was not affected. A delay in sexual maturation was also observed in the two-generation study with silver zinc zeolite however in this case it was considered secondary to the pronounced effects on bodyweights.

- Bodyweight is the primary driver behind sexual maturity, so body weight at puberty (unaffected by treatment) is the relevant parameter.

Section 10.10.3, Paragraph 7, Page 156.

Taken together, the effects observed with silver acetate and/or nanosilver (reduced fertility index, number of implantations, effects on sperm parameters, spermatogenesis and number of spermatogenic cells and delay in onset of puberty) are considered to clearly indicate an adverse effect on sexual function and fertility.

- See individual arguments above. There is no 'clear indication' of an adverse effect on sexual function or fertility. The only indication comes from the Sprando (2016) study (seen at the highest dose level of 40 mg/kg bw/d), but this was not replicated at higher dose levels in more reliable studies. Effects on sperm parameters are questionable and not of sufficient severity to cause effects on fertility.

Reliance on published data for AgNPs is not scientifically justified, with the DS themselves acknowledging that individual studies are not reliable.

Section 10.10.3, Paragraph 7, Page 156.

However, the relevance of data on nanosilver for the assessment of the silver ion and silver nitrate need to be considered. At least some of the effects noted in the studies could result from oxidative stress leading to apoptosis. This may be caused by the nanoparticle itself or the silver ions released. The silver ion has been shown to induce oxidative stress in studies performed with silver nitrate available in the open literature thus it is reasonable to assume that it is an intrinsic ability of the silver ion that is expressed also when dissolved from soluble salts like silver acetate and silver nitrate. Germ cells seem to be highly sensitive to this mechanism, but it is recognised that there may also be other modes of action for the effects observed.

- Published data for AgNPs do indeed point to oxidative stress as a mechanism underlying testicular toxicity. This may be an intrinsic property of the silver ion, but differences in the kinetics and deposition of silver need to be recognised. Boudreau noted clear differences in the morphology and distribution of silver deposits. Following dosing with AgNPs silver deposits were predominantly intracellular, whereas deposits following dosing with silver acetate (silver ion) were associated with extracellular membranes. This difference clearly has the potential to influence toxicity, particularly oxidative stress.

Section 10.10.3, Paragraph 8, Page 156.

It may also be discussed if nanoparticles of silver are distributed to and penetrate germ cells different from silver ions. The distribution of nanoparticles of silver as well as of silver nitrate was investigated in a 28-day study in rats (Van der Zande et al.). The results indicate a silver distribution pattern upon oral exposure to two different sizes of AgNPs and to AgNO3 with highest amounts in liver and spleen, followed by the testis, kidney, brain, and lungs, without differences in the distribution pattern between the two different AgNPs, or the AgNO3 exposed animals. The uptake of silver in blood and organs was higher in animals treated with AgNO3 compared to AgNP treated rats.

- This is not inconsistent with other data, but other studies note that the size and deposition of silver particles is different following oral dosing with soluble salts and AgNPs.

Section 10.10.3, Paragraph 8, Page 156.

However, when normalising the silver exposure dose in blood for soluble silver the difference in blood was much smaller. This indicates that the major part of silver in plasma is ionic silver released from the nanoparticles. Normalising for soluble silver exposure dose in organs resulted in similar silver contents between Ag<20 and AgNO3 in all organs except for testis and spleen in which the silver dose was higher for Ag<20. The authors thus conclude that AgNP only contributes to the silver concentration in these two organs and to a much lower extent, a result stated to be in contrast with a different study indicating that not only soluble silver but a significant fraction of AgNPs contribute to silver in organs.

- The study therefore confirms that testicular concentrations of silver are proportionally higher following exposure to an AgNP compared to a soluble salt. Taking into account the fact that the study only investigated a single type of AgNP and also considering the variability in the results of published studies, this casts further doubt on the reliance on AgNP data.

Section 10.10.3, Paragraph 8, Page 156.

Nevertheless, of significance for this assessment is the detection of silver from both nanoparticles of silver and AgNO3 in the testis meaning that it is reasonable to assume that effects discussed in this section are not specific to nanoparticles but also relevant for effects of ionic silver from salts administered via the oral route.

- Refer to differences in deposition between silver salts and AgNPs, differences in testicular exposure, and the high level of variability in testicular toxicity reported for AgNPs.

Section 10.10.3, Paragraph 8, Page 156.

This is also confirmed by the new EOGRTS data on silver acetate.

- The EOGRTS shows borderline/unconvincing effects on sperm parameters, in the absence of testicular toxicity, and without any consequence for fertility in the EOGRT study.

Section 10.10.3, Last Paragraph, Page 157.

The data available on silver acetate and nanosilver indicate that the silver ion has the ability to cause adverse effects on sexual function and fertility possibly by a mechanism involving oxidative stress. According to the original report for the silver acetate study and the published studies with nanosilver there were no marked general toxicity indicating that effects were "a non-specific consequence of the other toxic effects".

- Not sure which silver acetate study is referred to here? The only study showing effects on sexual function and fertility is the 1-generation study of Sprando (2016), which is inconsistent with more robust studies using higher dose levels. The EOGRTS with silver acetate does not show any effects on fertility, the effects on 'sexual function' are limited to borderline effects on sperm parameters.

Section 10.10.3, Last Paragraph, Page 157.

There is no reason to expect a lower sensitivity of humans to these effects and therefore criteria for classification in category 1B, H360F are considered fulfilled.

- There are no data indicating lower sensitivity of humans, but the available animal data are not sufficiently clear for **silver nitrate** to trigger classification in Category 1B.

Section 10.10.4 – Adverse effects on development (proposed Category 1B)

Comments on DS comparison with the CLP criteria

Section 10.10.6, Paragraph 1, Page 212.

Foetal/pup mortality is also indicated at the low dose in a study performed with nanosilver (Philbrook et al.) however due to the limited dosing period (a single dose on GD9) and the lack of surface coating which possibly prevents higher exposures to nanosilver and/or silver ions, the result from this study is not comparable. Likewise, the results from Yu et al. in which rats were administered high doses of nanoparticles during gestation days 6-20 only are not comparable with the results from silver chloride (exposure over the entire gestation period) and silver acetate/silver zinc zeolite/silver zirconium hydrogen phosphate studies that were performed with continuous exposure during pre-mating, gestation and lactation.

- Using data from studies with AgNPs is highly questionable. The study of Philbrook et al (2011) used single dose levels of 10, 100 and 1000 mg/kg bw. A statistically significant effect was seen only at 10 mg/kg bw/d (not at 100 or 1000 mg/kg bw), a dose level significantly below those of SCAS or soluble salts causing similar effects. Furthermore, studies with other forms of silver show that repeated dosing over the entire gestation period (at least) is required to cause effects. The results of the Philbrook study therefore have to be questioned. The study of Yu *et al*. also referred to by the DS does not show any effects at oral dose levels of up to 1000 mg/kg bw/d administered over most (but not all) of the gestation period. The results of this study are interpreted in light of the responses seen with other forms of silver, but the obvious lack of consistency with the Philbrook study is not acknowledged.

Section 10.10.6, Paragraph 1, Page 212.

Considering that the plausible mechanism of toxicity presented is a gradual decrease of active ceruloplasmin from plasma of dams, it can be questioned if silver toxicity would be detected in the standard developmental toxicity studies unless treatment is continuous during the entire gestation period. Therefore, one or two-generation studies with continuous exposure during pre-mating, gestation and lactation seem more appropriate to detect this type of toxicity in the foetus/pup. A possible explanation why effects seen in the two-generation study performed with silver sodium hydrogen zirconium phosphate were less pronounced despite a similar or even higher exposure to silver ions compared to silver acetate could be the difference in administration route (in drinking water and diet respectively) or due to normal variation between studies. In the case of silver zinc zeolite the presence of zinc could possibly also replace copper in ceruloplasmin and that could hypothetically contribute to the observed effects if this was the mechanism or one of the mechanisms causing the developmental effects. However, silver zinc zeolite is not the only silver- containing and –releasing substance that caused certain developmental effects and therefore zinc in not expected to be the critical contributor for the observed developmental effects by silver zinc zeolite.

- The postulated mechanism is likely. Variability between studies can be explained by systemic exposure to silver (a function of silver content, release and solubility), duration of exposure, and the level of dietary copper. Exposure to high levels of zinc has been shown to reduce circulating copper (but not ceruloplasmin levels), so will contribute to the effects seen in the 2-generation study with SZZ which were considered sufficient to only require Category 2 classification.

Section 10.10.6, Paragraph 2, Page 212.

Dietary administration of silver acetate at dose levels of 40, 80 and 120 mg/kg/day was associated with a number of effects including: F1 mortality at 120 mg/kg/day; F0/F1 red blood cell parameters at all dose levels; F1 offspring survival at 120 mg/kg/day; F1 offspring body weight at 80 or 120 mg/kg/day; F1 neurobehaviour/sensory function at 80 or 120 mg/kg/day; motor activity for F1 males and females at 80 or 120 mg/kg/day;

- The dose level of 40 mg/kg bw/d is from Sprando (2016); the effects are disputed as they are different to those seen at higher dose levels in the (more reliable) EOGRT study.

Section 10.10.6, Paragraph 3, Page 213.

In addition to substance-related increases in mortality rates of foetuses/pups by silver acetate (EOGRTS), silver chloride (Shavlovski, 1995), silver zinc zeolite, silver acetate and nanosilver (Philbrook et al., 2011) and to some extent by silver sodium zirconium hydrogen phosphate as well as increases in mortality rates

of adolescences and young adults (possibly also being a consequence of developmental exposure) by silver acetate (EOGRTS), silver zinc zeolite in the 2-generation study.

- Data for AgNPs should be discounted; in particular, the Philbrook study which is anomalous and cannot be relied upon.

Section 10.10.6, Paragraph 3, Page 213.

There were also other developmental effects including significant brain damage (by silver acetate in the EOGRTS, by nanosilver in Fatemi et al., 2013), increase in relative brain weight (by silver acetate in the EOGRTS, by silver zinc zeolite, effects on neurobehavior/sensory function (in the EOGRTS, DNT cohort)

- Data for AgNPs should be discounted. Brain effects were seen in the EOGRTS with silver acetate, but these would seem to be mild and not to constitute 'significant damage' sufficient to trigger classification in Category 1B. Increases in relative brain weight have to be considered in light of body weight changes (as brain weight is conserved). Effects on behaviour in the EOGRTS are acknowledged, but again should be viewed in light of general toxicity including bodyweight effects.

Section 10.10.6, Paragraph 3, Page 213.

…enlargement of heart/increase in heart weight (by silver acetate in the EOGRTS), by silver zinc zeolite and cryptorchidism (by silver chloride in Doc IIIA, 6.8.1(03), Shavlovski, 1995)

- Effects on the heart are secondary to haematological effects. For SZZ, RAC consider the enlargement of hearts reported at the mid dose in F2 pups (5/27 males and 4/26 females) appeared in the presence of mild maternal toxicity (mainly hydronephrosis and haematological alterations). The effects also appeared in F1 pups at the highest dose (6/14 males and 6/18 females), in the presence of excessive maternal toxicity. The effects were not discounted but were seen as relevant for classification of SZZ as toxic to development Category 2.

- Cryptorchidism is not seen in any other study. It is notable that the absence of maternal toxicity seen in the Shavlovski (1995) study was reported to occur in the presence of total copper depletion in serum. In contrast, the EOGRT range finder study dosed at 320 mg/kg bw and 160 mg/kg bw resulted in a significant reduction in serum copper that coincided with general maternal toxicity that led to premature termination for welfare reasons. The Shavlovski results appear questionable and suggest incomplete reporting of toxic effects.

Section 10.10.6, Paragraph 4, Page 213.

Neurodevelopmental toxicity is also reported in the following published literature studies on silver nanoparticles. Wu et al. (2015) prenatally dosed offspring via mothers every two days from GD10 to GD18 ip to uncoated silver nanoparticles and showed histopathological changes with hippocampal neuronal cell loss along with impaired spatial learning and memory ability tested in Morris water maze test in rat offspring at postnatal day 35. In Ghaderi et al. (2015). NMRI mice had been treated subcutaneously once every three days from gestation day 3 until delivery, by 0, 0.2 and 2 mg/kg of bodyweight of silver nanoparticles. Spatial memory, passive avoidance learning, stress, anxiety-like behaviours and locomotor activities were assessed in adult offspring. Prenatal exposure to silver nanoparticles significantly impaired the cognitive behaviour in the Morris water maze. Also, the number of defecations and leanings in the open field assay and number of passages in the light-dark box were greater in groups prenatally exposed to silver nanoparticles. In Yin et al. (2015), an intranasal application of citrate stabilised silver nanoparticles to neonatal SD rats for 14 weeks showed that silver nanoparticles caused cerebellar ataxia

like symptom in these rats, evidenced by dysfunction of motor coordination and impairment of locomotor activity. Observation of cerebellum sections revealed destruction of the cerebellum granular layer.

- Question the reliance on data for AgNPs

Section 10.10.6, Paragraph 5, Page 213.

One plausible mechanism for silver developmental toxicity, i.e. silver interfering with copper binding to ceruloplasmin and thereby reducing the availability of copper, iron or perhaps both metals to the foetus, is supported by the copper analysis of F0 and F1 pups in the EOGRTS and in the F2 pups in the silver zinc zeolite study. Studies also show reduced ceruloplasmin activity. This information indicates that the developmental effects are caused by a specific mechanism rather than being a secondary non-specific consequence of toxicity in the mother. Based on the effects observed, pups seems to be more sensitive than dams to the adverse effects caused by silver. Since ceruloplasmin has the same function in humans, this potential mechanism cannot be considered irrelevant for humans. In addition, there is no evidence raising a doubt of human relevance of the adverse developmental effects for humans.

- The mechanism is accepted as a plausible in the RAC opinions for SZZ and silver. The mechanism is likely to be relevant to humans, so indeed cannot be discounted. Reduced offspring viability was previously considered to support Category 2 classification.

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