# Additional information report

## For a Substance under Harmonised Classification and Labelling Process

## Substance Name: Silver

**EC Number: 231-131-3**

**CAS Number: 7440-22-4**

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# 1 INTRODUCTION

During the CLH process of silver, ECHA received a copy of the following newly performed studies:

- Silver Acetate: Preliminary Reproductive Performance Study in the Sprague Dawley Rat by Dietary Administration (Renaut et al., 2022)

- Silver Acetate: Extended One Generation Reproductive Toxicity Study in the Sprague Dawley Rat by Dietary Administration (Renaut et al., 2022)

To assure an efficient and transparent assessment of the new information provided by the data submitter and giving parties concerned an opportunity to comment, it is the responsibility of the data submitter to elaborate non-confidential detailed study summaries and explain the relevance of the provided information for the classification at issue. To facilitate the task of the data submitter, ECHA has compiled a format for Additional information report.

Every study should be presented individually. The relevance of each study for the classification at issue should be clearly presented. A non-confidential detailed study summary and the results of the study should be reported transparently and objectively as in the original data source without any interpretations by the data submitter. A detailed study summary includes test type (e.g. test guideline followed and deviations from the guideline, the GLP and quality assurance status of the study, identity of the test substance (including degree of purity), information about the test subjects, route of administration, mode of exposure and numerical results. If read-across to structurally or mechanistically similar substances is used a justification for using data from a different substance should be provided and, if known, calculations to convert dose/concentration levels from the test substance to the substance for which CLH is proposed should be presented.

## 2 TOXICITY TO REPRODUCTION STUDIES

The Extended One-Generation Reproductive Toxicity study assesses the influence of silver acetate on reproductive performance when administered continuously via the diet to Sprague Dawley rats in an OECD 443 EOGRTS. A pre-mating exposure period of ten weeks for males and females was performed. Cohorts of F1 animals were used to assess the potential for systemic toxicity, and potential effects on sexual maturation and oestrus cycle, developmental neurotoxicity and developmental immunotoxicity. The appropriate species (rats) and route of exposure is adequate.

The dose range finding study performed with silver acetate assists in the dose level selection for the main EOGRTS mentioned above. A pre-mating exposure period of four weeks for males and females was selected to optimize assessment of any potential effects on reproductive outcomes.

These studies assess the potential toxicity of silver acetate (AgAc) on reproduction and development of Sprague Dawley rats exposed via diet. These studies are shared in the context of the reproductive toxicity discussion by providing additional information regarding reproductive toxicity of silver ions ($Ag^+$).

**Study references:**


1. Renaut et al. (2022) Preliminary Reproductive Performance Study in the Sprague Dawley Rat by Dietary Administration

2. Renaut et al. (2022) Silver Acetate: Extended One Generation Reproductive Toxicity Study in the Sprague Dawley Rat by Dietary Administration


## 3 Justification for using data from different substance (Silver Acetate)

Silver acetate was proposed because it is a silver (1+) salt, which is soluble in water – it possesses a solubility value of approximately 10 g/L at 20°C (Merck, 2013). It has been commonly utilised in a variety of published toxicology studies as a soluble silver (1+) reference compound. Ag acetate is known to exhibit satisfactory bioavailability via the oral route. Testing outcomes from a study utilising silver acetate are anticipated to be fully applicable for read-across to ionic silver (Ag+) donor compounds such as silver nitrate and disilver(I) oxide but not to silver metal (powder and massive). For Ag metal (massive and powder), a weight of evidence is supported based on *in-vivo* comparative toxicokinetic data in the dossier.

## 4 RELEVANCE OF STUDIES

The reproductive toxicity assessment of silver metal (massive and powder) (AgMP) is based on the EOGRT study (+ dose range finding) performed with silver acetate (AgAc). However, a correction of the findings in the study with AgAc is required based on a demonstrated lower bioavailability of Ag metal (massive and powder) vs soluble Ag salts (like AgAc).

It is assumed that systemic toxicity of silver substances is driven by $Ag^+$ (silver ions) as the primary species for systemic circulation and relevant for tissue exposure, and hence hazard assessment. Thus, a very low bioavailability leads to a very low internal concentration of $Ag^+$ (toxicophore), to a lack of biological interaction and hence the absence of adverse outcome at similar dosing levels (expressed as Ag concentration) in comparison with highly bioavailable silver compounds.

The bioavailability of AgAc via oral route is consistent between independent estimates presented in the scientific literature (Barraclough and Cotton 2017, Boudreau et al. 2012, Park et al. 2011 and Van der Zande et al. 2012) and the comparative *in-vivo* TK study – in the order of 3-5%. In comparative terms, silver metal powder (AgMP) is substantially less absorbed. Based on matched dose assessments performed in the *in-vivo* TK study (Melvin et al., 2021 and Charlton et al., 2021, the extent of systemic exposure was about 10 to 30-fold lower in the case of AgMP vs AgAc. Unlike of situation of AgAc, the degree of uptake is not linear as the amount of administered of AgMP was increased up to a limit dose, but instead there was evidence of absorption plateauing (**figure 1**). As a generic observation, the following trend in bioavailability is being observed: AgAc / $AgNO_3$ >> AgNP >>> AgMP.

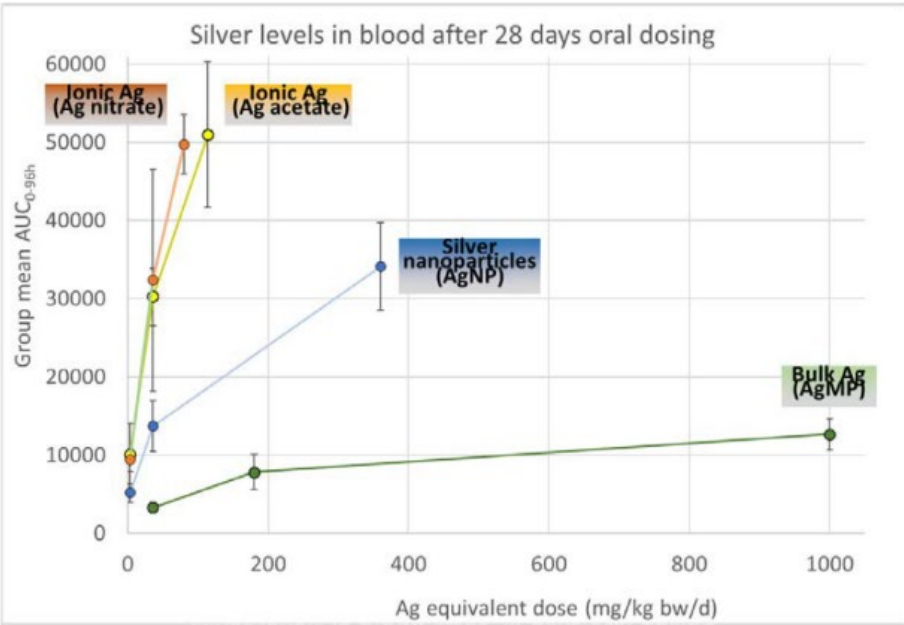


**Figure 1. Marked differences exist in the extent of absorption observed for ionic Ag (tested as Ag acetate and Ag nitrate) versus that of bulk Ag (sub-micron Ag metal powder, 'AgMP').**

*Results are for female animals receiving the test items (very similar findings were also obtained in the case of males).*

*Data are expressed as Ag equivalent dose. Standard deviations are added.*

Therefore, the toxicokinetic findings strongly suggest that the direct read-across of mammalian toxicity data with soluble silver salts (like silver acetate and silver nitrate) and nanosilver to silver metal (powder and massive) is <u>not</u> justified (based on their respective toxicokinetic profiles, presented in **figure 1).**

This new evidence demonstrates that oral intake of AgMP results in markedly lower absorption, distribution and systemic tissue/organ exposure to silver than more bioavailable forms like AgAc.

Therefore, the Toxicokinetic / bioavailability / $Ag^+$ release parameter (Melvin et al., 2021; Charlton et al., 2021) is considered as key piece of evidence in the weight of evidence reasoning for the repeated dose toxicity of silver metal (massive and powder) by oral route.

Moreover, the European Precious Metals Federation (EPMF) has provided an expert opinion regarding possible mode of action mechanisms underlying the observed effects. The opinion is attached to this document (**Annex 3**).

**In summary:**

1) Evidence exists from rodent models that administration of ionic Ag can cause a copper deficiency (Cu-D) state, through Ag+-induced deformation of the key Cu transporter protein ceruloplasmin (Cp). Treatment with ionic Ag, e.g. for 4 weeks or more, has been shown to cause loss of Cp activity and perturbation of Cu homeostasis.

2) In the EOGRTS with silver acetate, serum Cu measurements performed in F0 and F1 rats clearly showed a treatment-related pattern of Cu level depression, with serum Cu concentrations inversely related to increasing blood Ag concentration. At the dose levels associated with the main treatment-related findings in the EOGRTS, the deficiency state can be categorised as either moderately severe or moderate in its degree.

3) The following treatment-related effects were observed in F0 and F1 rats in the EOGRTS: (i) haematological and biochemical shifts (mainly anaemia, increased plasma alkaline phosphatase activity and hypercholesterolemia); (ii) organ weight changes indicative of cardiac hypertrophy and thymic involution; (iii) changes in littering indices (reductions in live birth rate and pup viability); (iv) pup growth rate depression, and development of poor condition or failure to thrive; (v) brain myelopathy, cell loss, and diminution of the size of the hippocampus; and (vi) deficits in motor function and neurobehavioral parameters. As described in detail in the attached expert opinion, all these effects are related to indirect Cu-D mediated by Ag+ exposure.

4) EPMF investigated the Cu status of rats treated with a sub-micron silver metal powder (AgMP) during a recent comparative OECD TG 417 TK study. Findings were compared to those of animals which received an ionic Ag reference form (silver nitrate, with a proven similar TK profile than silver acetate). Even at a limit dose (1000 mg Ag/kg bw/d), AgMP did not cause Cu-D in rats following 28 days treatment (p.o.), in contrast with silver nitrate where circulating Cu levels were already depressed as from 35 mg Ag/kg bw/d. It should be noted that 1) the highest tested Ag equivalent dose level for AgMP was more than 12 times that for silver nitrate and 2) steady-state conditions for Ag are reached after repeated dosing for a period of about two weeks.

In conclusion, based on comparative TK data coupled to Cu serum determinations, it is unlikely to attain a condition of sufficient Cu depletion in the case of orally administered silver metal (massive and powder) at relevant dose levels for hazard classification (up to limit dose).

## 5 Detailed study summary and results of DRF study (Renaut et al. 2021)

Preliminary Reproductive Performance Study in the Sprague Dawley Rat by Dietary Administration

### Information substance identify

**Test item:** Silver Acetate
**CAS number:** 563-63-3
**EC number:** 209-254-9
**Intended use**: Chemical feed stock
**Appearance:** Colourless to pale-white crystalline powder
**Storage conditions**: room temperature and protected from light
**Expiry date**: 22 October 2021
**Purity**: > 99.5%
**Molar mass**: 166.91 g/mol (107.87g/mol Ag)
**Solubility**: Aqueous (room temperature): 10.2g/mL

### Study summary

In the F0 generation, four groups of 12 male and 12 female rats received Silver Acetate (AgAc) orally, via the diet, at target dose levels of 4, 40, 80, 160 and 320 mg AgAc/kg bw/day. Males were treated for 29 days before pairing and until necropsy after litters had weaned (after at least 65 days of treatment). Females were treated for 29 days before pairing, throughout pairing, gestation and until necropsy on Day 21 of lactation. In the F1 generation, three groups of 10 males and 10 females were treated at target dose levels of 4 or 40 mg/kg bw/day from weaning during late lactation to their scheduled termination on Days 31-33 of the F1 generation [at nominal Day 59-61 (+/- 2 days) of age] following completion of sexual
maturation. A similarly constituted Control group received the vehicle, (untreated diet) for the same duration for each generation.

During the F0 generation, clinical condition, body weight, food consumption, oestrous cycles, pre-coital interval, mating performance, fertility, gestation length, exposure assessment and biomarkers, organ weights and macroscopic and microscopy pathology investigations were undertaken. In addition, haematology and blood chemistry investigation s were undertaken for males at 4, 40, 160 and 320 mg/kg bw/day and females at 4 and 40 mg/kg bw/day.

The clinical condition, litter size and survival, sex ratio, body weight, exposure assessment and biomarkers, organ weights and macropathology for all offspring were also assessed.

For the selected F1 generation at 4 and 40 mg/kg bw/day clinical condition, body weight, food consumption, sexual maturation, exposure assessment and biomarkers, organ weights and macroscopic pathology investigations were undertaken.

**Results**

**Biomarkers**

Ceruloplasmin activity was observed to decrease with increasing doses of Silver Acetate at 4, 40, 160 and 320 mg/kg bw/day for F0 male and female animals on Day 12, F0 males on Day 43 and female animals on Gestation Day (GD) 6. F1 Male and female animals followed the same trend on Day 21 at 4 mg/kg bw/day and 40 mg/kg bw/day; male and female animals dosed at 80 mg/kg bw/day showed results inconsistent with this trend.

There was little impact on Glutathione Peroxidase (GPx) activity observed following administration of Silver Acetate at dose levels up to and including 320 mg/kg bw/day in male rats. At 320 mg/kg bw/day, female rats showed a decrease (37-42% lower than in Controls) in activity at Day 12 and GD6. Both male and female animals at 80 mg/kg bw/day showed low GPx activity.

Blood levels of silver generally increased with increasing target dose levels; no detectable levels of silver were found in the Control samples.
Silver tissue concentrations were below detectable levels in the brain samples obtained from Control offspring on both Day 4 and Day 21 of age. Silver levels in the rat tissue samples increased with increasing target dose levels, with the highest concentrations detected in the milk pellets obtained from offspring on Day 4 of age.
Serum levels of copper and selenium tended to decrease as the target dose level increased; the observed effect was more marked for copper.
There was evidence for a dose-related decrease in copper levels in the testes for F0 males and a non-dose related increase in selenium levels in the ovaries for F0 females. There was no clear effect of treatment on the ovarian copper levels, or the testis selenium levels.

**F0 Mortality**

Between completion of parturition and Day 2 of lactation four females at 320mg/kg bw/day and two at 160 mg/kg bw/day were killed following total litter loss with offspring either found dead, missing (presumed cannibalized) or killed for welfare reasons. As littering progressed it became apparent that these dose levels were not tolerated and the remaining females in these dose groups were terminated on welfare grounds; at the time of termination the females were between GD20 and LD4.

Macroscopic examination of the F0 females at 160 and 320 mg/kg/day revealed a number of abnormalities including abnormal colour/content of the GI tract, abnormal coloured pancreas and mesenteric lymph nodes.

At macroscopic examination the mammary tissue appeared pale and inactive for two females that received 160 mg/kg/day (no. 140 total litter loss on Day 1 of lactation, and no. 147 killed on Day 1 of lactation) and for four females that received 320 mg/kg bw/day

(nos.151,154 and 157 with total litter loss on Days 1/2 of lactation and no.159 killed on Day 1 of lactation).

Post-implantation survival and the live birth index were low at 160 and 320 mg/kg/day resulting in low litter sizes at this dose levels.

The offspring body weight for those assessed on Day 1 of age was low when compared with Controls and examination of the decedent offspring predominately revealed an absence of milk in the stomach.

**F0 Generation**

The target dose levels on this study were 4, 40, 80, 160 and 320 mg/kg/day and the mean achieved dose levels were as follows:

- F0 males: 3.90, 38.7, 76.0, 152, and 308mg/kg bw/day (95-98% of intended)
- F0 females before pairing: 4.52, 44.6, 94.6, 177 and 378 mg/kg bw/day (111-118% of intended)
- F0 females during gestation: 4.97, 50.4, 90.6, 198 and 338 mg/kg bw/day (106-126% of intended)
- F0 females during lactation: 5.74, 54.0 and 92.1 mg/kg/day (115-144% of intended) at 4,40 and 80 mg/kg bw/day, respectively.

Routine physical examination of males from the start of treatment up to termination and for females before pairing, after mating and during lactation did not reveal any findings that could be attributed to administration of silver acetate.

Overall, the mean body weight gain for males at 320 mg/kg bw/day was markedly low at approximately 20% of Controls, resulting in low mean body weight at the end of the treatment period.

The overall body weight gain of F0 females during the four-week pre-pairing treatment period was low at the target dose of 320 mg/kg bw/day at approximately 38% of Controls. From Day 3 of gestation body weight gain for females at 320 mg/kg bw/day was low resulting an overall body weight gain that was approximately 42% of Controls and a low mean body weight on GD20.

Assessment of body weight gain during lactation which was restricted to females at the target dose levels of 4, 40 and 80 mg/kg bw/day showed poor body weight performance during. Days 4-7 and Days 11-14 for females receiving 80 mg/kg bw/day.

For most food consumption phases F0 males at 320 mg/kg bw/day showed statistically significantly low mean values when compared with Controls ($p<0.01$).

Before pairing females at 320 mg/kg bw/day showed periods of low food consumption and subsequently females at this target dose level showed consistently low food consumption throughout gestation.

Assessment of food consumption during lactation which was restricted to females at the target dose levels of 4, 40 and 80 mg/kg bw/day showed no adverse effects of treatment.

Assessment of oestrous cycles, pre-coital interval, mating performance and fertility assessment did not show any adverse effects of treatment.
At 40 mg/kg/day there was a shift towards a slightly longer gestation length ($p<0.05$) but the gestation index was 100%.

Assessment of the gestation length at 160 mg/kg bw/day and 320 mg/kg bw/day was limited to females that littered before the groups were terminated prematurely and showed a shift towards a slightly longer gestation length.

At scheduled termination males at target dose levels of 160 or 320 mg/kg bw/day showed statistically significantly low haematocrit, haemoglobin level, mean cell haemoglobin, mean cell haemoglobin concentration and mean cell volume ($p<0.01$); these was no evidence for a dose response. Males at 40 mg/kg bw/day also showed a low mean cell haemoglobin ($p<0.05$). Platelet counts for males that received 40, 160 or 320 mg/kg bw/day were slightly high ($p<0.05$; $p<0.01$).

Alkaline phosphatase activity and cholesterol levels for males and females at 40 mg/kg bw/day and for males at 160 and 320 mg/kg bw/day were high ($p<0.05$; $p<0.01$, except female cholesterol); at 4 mg/kg bw/day the mean cholesterol levels for males was also slightly high ($p<0.05$). Other minor differences included: high alanine amino transferase activity and calcium for males at 320 mg/kg bw/day ($p<0.01$); slightly low chloride levels for males at 320 mg/kg bw/day ($p<0.01$); high phosphorous levels for females at 40 mg/kg bw/day ($p<0.05$); high albumin/globulin ratio for females at 40 mg/kg bw/day ($p<0.05$).

At scheduled termination the body weight relative liver weight for males that received target dose levels of 160 or 320 mg/kg bw/day were high when compared with Controls ($p<0.01$); the body weight relative liver weight at 80 mg/kg bw/day also looked slightly high for male and female animals but in the absence of a concurrent control for this dose group a true assessment could not be made.

At scheduled termination, Silver Acetate-related dark coloration of the liver was observed in males administered 40, 160 and 320 mg/kg bw/day and correlated with pigment deposition observed at microscopic examination. The same macroscopic lesion was observed in females administered 40 and 80 mg/kg/day.

Silver acetate-related dark content of cecum was observed in males administered 160 and 320 mg/kg bw/day and in females administered 80 mg/kg bw/day; dark content of rectum was observed in males administered 320 mg/kg/day and in females administered 80 mg/kg bw/day.

Silver acetate-related abnormal colour of the pancreas and mesenteric lymph nodes was observed in males administered 40, 160 and 320 mg/kg bw/day and in females administered 40 and 80 mg/kg bw/day.

Silver acetate-related dark coloration of the uterus, kidney, thymus, urinary, bladder and salivary gland was observed in females administered 80 mg/kg bw/day; one female at 40 mg/kg bw/day exhibited dark mandibular salivary glands and uterus.

Pigment was observed in Kupffer cells and to a lesser extent in hepatocytes and in the vascular walls of the liver of males administered 160 and 320 mg/kg bw/day and correlated with macroscopic dark liver colour. Centrilobular hepatocellular hypertrophy was observed in males administered 160 and 320 mg/kg bw/day and correlated with increased liver weight.

**F1 Litter Responses**

**Group 4 (160 mg/kg/day) and Group 5 (320 mg/kg/day) were terminated prematurely late gestation/late lactation after four total litter losses at 320 mg/kg/day and two at 160 mg/kg/day. Assessment of litter responses was therefore restricted to target dose levels of 4, 40 or 80 mg/kg/day.**

There was no clear effect of treatment on the clinical condition of offspring at 4, 40 or 80 mg/kg/day; one litter at 80 mg/kg/day no. 174 was observed with whole body pallor from Day 7 to Day 14 of age and the coat was recorded as patchy on Days 20/21 of age.

Litter size, offspring survival, sex ratio and ano-genital distance showed no adverse effects of treatment at target dose levels up to and including 80 mg/kg bw/day.

Offspring body weight on Day 1 of age and subsequent body weight gain up to Day 7 of age was unaffected by parental treatment at dose levels up to and including 80 mg/kg bw/day. At 4 and 40 mg/kg bw/day offspring bodyweight gain from Days 7-11 of age was slightly low ($p<0.05$) when compared with Controls. Offspring body weight at 80 mg/kg bw/day did not show any signs of overt toxicity but in the absence of concurrent Control assessment was limited.

Offspring Brain Weight on Day 21 of age was unaffected by administration of Silver Acetate at dose levels up to and including 80 mg/kg bw/day.

Macroscopic examination of offspring that died prior to scheduled termination did not reveal any findings at dose levels up to and including 80 mg/kg bw/day that could be attributed to administration of Silver Acetate.

At scheduled termination of unselected offspring on Day 21 of age macroscopic findings were restricted to one litter at 80 mg/kg/day (litter no. 174) where all offspring exhibited a pale liver and generalized hair loss, one offspring had a diaphragmatic hernia, and one offspring had a liver mass on the median liver lobe.

Detailed brain histopathology of F1 offspring at Day 21 of age did not reveal any pathological changes or developmental abnormalities.

**F1 Generation**

**Group 4 (160 mg/kg bw/day) and Group 5 (320 mg/kg bw/day) were terminated prematurely late gestation/late lactation after four total litter losses at 320 mg/kg bw/day and two at 160 mg/kg bw/day. Assessment of F1 responses was therefore restricted to phase 1 animals receiving 4 or 40 mg/kg bw/day; litters at 80 mg/kg bw/day (Group 7) were terminated on Day 21 of age.**

The target dose levels on this phase of the study were 4 and 40 mg/kg bw/day and the achieved dose levels were as follows:

- F1 males: 4.5 and 45.0 mg/kg bw/day (113% of intended)
- F1 females: 4.3 and 46.2 mg/kg bw/day (108-116% of intended)

There were no clinical signs at routine physical examination that could be attributed to administration of Silver Acetate at 4 or 40 mg/kg bw/day.

At weaning on Day 21 of age mean body weight was similar across the groups for both males and females.

The mean body weight for selected males on Day 1 of the F1 generation was similar across the groups, however the overall gain for males from Day 1 to Day 29 was low at 4 and 40 mg/kg bw/day. The resultant mean body weight at these target dose levels were low when compared with Controls.

On Day 1 of the F1 generation the mean bodyweight for females at the target dose of 40 mg/kg bw/day was slightly low and this difference in mean body weight persisted up to Day 29 of the F1 generation. Overall the body weight gain for females at 40 mg/kg bw/day was slightly low at 90% of Controls but this difference did not attain statistical significance.

Food consumption for selected F1 animals from Days 1 to 29 of the F1 generation was unaffected by administration of Silver Acetate at 4 or 40 mg/kg bw/day.

The age at completion of sexual maturation for both males and females was unaffected by administration of Silver Acetate at target dose levels of 4 and 40 mg/kg bw/day. The mean body weight on the day of completion of preputial separation was slightly but statistically significantly low for males receiving 4 or 40 mg/kg bw/day; females at 40 mg/kg bw/day also showed slightly low mean body weight at vaginal opening but this difference did not attain statistical significance.

The absolute mean brain weight for selected F1 females that received 40 mg/kg bw/day was slightly but statistically significantly low when compared with Controls ($p<0.05$); this difference was not evident for males at this dose level.

Macroscopic examination of F1 animals at scheduled termination following attainment of sexual maturation did not reveal any abnormality.

## Conclusion

Based on the findings in this study, dose levels of 160 and 320 mg/kg bw/day were not tolerated and were considered unsuitable for the subsequent OECD 443 study. As no significant adverse effects were observed in parental animals or in F1 pups (to weaning) at 80 mg/kg bw/day, the optimal high dose level for the OECD 443 study is between these levels, at 120 mg/kg bw/day.

## 6 Detailed study summary and results of EOGRTS study (Renaut et al. 2021)

Silver Acetate: Extended One Generation Reproductive Toxicity Study in the Sprague Dawley Rat by Dietary Administration

**Information substance Identity:**

**Test item**: silver acetate
**CAS number**: 563-63-3
**Intended use**: Chemical feed stock
**Appearance**: Colourless to pale-white crystalline powder
**Storage condition**: room temperature and protected from light
**Expiry date**: 22 October 2021
**Purity** > 99.5%
**Molar mass:** 166.91 g/mol (107.87 g/mol Ag)
**Solubility**: Aqueous (room temperature): 10.2 mg/mL

**Detailed study summary and results:**

The purpose of this study was to assess the influence of Silver Acetate (purity ≥ 99.99% - silver content 64.59%) on reproductive performance when administered continuously via the diet to Sprague Dawley rats in an OECD 443 Extended One Generation Reproductive Toxicity Study. Cohorts of F1 animals were used to assess the potential for systemic toxicity, and potential effects on sexual maturation and oestrus cycles, developmental neurotoxicity and developmental immunotoxicity.

The study was designed to meet the requirements of the following guideline:

- Organization for Economic Co-operation and Development Testing of Chemicals Guideline No. 443: (Adopted 25 June 2018).
- The study was conducted in accordance with the requirements of current, internationally recognized Good Laboratory Practice Standards.

In the F0 generation, three groups of 25 male and 25 female rats received Silver Acetate at target dose levels of 40, 80 or 120 mg/kg bw/day orally, via the diet. Males were treated for ten weeks before pairing, up to necropsy after litters were weaned. Females were treated for ten weeks before pairing, throughout pairing up to necropsy on Day 28 of lactation. The F1 generation was treated from weaning to their scheduled termination (relevant to each cohort) at the same dose levels and volume-dose as the F0 generation, with exception on animals at 120 mg/kg bw/day in Cohorts 1A and 1B which were terminated prematurely on welfare grounds at approximately 10 weeks of age rather than 13-14 weeks of age.

| Cohort | Group | Number of animals | | Approximate age at termination |
|---|---|---|---|---|
| | | Male | Female | |
| 1A | 1 | 20 | 20 | 13 weeks |
| | 2 | 20 | 20 | 13 weeks |
| | 3 | 20 | 20 | 13 weeks |
| | 4 | 23 | 20 | 10 weeks |
| 1B | 1 | 20 | 20 | 14 weeks |
| | 2 | 20 | 20 | 14 weeks |
| | 3 | 20 | 20 | 14 weeks |
| | 4 | 17 | 18 | 10 weeks |
| 2A | 1 | 10 | 10 | PND75 |
| | 2 | 10 | 10 | PND75 |
| | 3 | 10 | 10 | PND75 |
| | 4 | 10 | 10 | PND75 |
| 2B | 1 | 10 | 10 | PND22 |
| | 2 | 10 | 10 | PND22 |
| | 3 | 10 | 10 | PND22 |
| | 4 | 10 | 10 | PND22 |
| 3 | 1 | 10 | 10 | PND60 |
| | 2 | 10 | 10 | PND60 |
| | 3 | 10 | 10 | PND60 |
| | 4 | 10 | 10 | PND60 |

A similarly constituted Control group received untreated basal diet, at the same volume dose and throughout the same relevant period as the treated groups. For the F0 generation data were recorded on clinical observations, body weight, food consumption, water consumption (by visual assessment), oestrous cycles, mating performance and fertility, gestation length and parturition observations and reproductive performance. Clinical pathology (hematology and blood chemistry) and thyroid hormone analysis, blood silver analysis, serum copper/selenium analysis, sperm assessment, organ weight and macroscopic pathology and microscopic pathology investigations were performed.

For F1 offspring, clinical condition, litter size and survival, sex ratio, body weight, ano-genital distance, organ weights and macropathology were assessed. Nipple counts were performed on male offspring on Day 13 of age. Serum samples that were collected from litters on Day 4 of age were analysed for serum copper/selenium levels and blood samples were analysed for silver levels. Serum samples from selected offspring on Day 22 of age were analysed for thyroid-related hormones and serum copper/selenium levels, and blood samples were analysed for silver levels.

**At weaning the F1 generation was split into five cohorts:**

For F1 Cohort 1A, data were recorded on clinical condition, body weight, food consumption, sexual maturation and oestrous cycles. Clinical pathology (hematology, blood chemistry, cardiac troponin, and urinalysis) and thyroid-related hormones, silver blood levels, serum copper/selenium levels sperm assessment, ovarian follicle and corpora lutea counts, organ weight, macroscopic pathology, full microscopic pathology and spleen immunophenotyping investigations were performed.

For F1 Cohort 1B, data was recorded on clinical condition, body weight, food consumption, sexual maturation and oestrous cycles. Silver blood levels and copper/selenium serum levels were assessed. Organ weight and macroscopic pathology investigations were performed.

For F1 Cohort 2A, data was recorded on clinical condition, body weight, food consumption and sexual maturation. Neurobehavioral screening, brain weight, brain morphometry, macroscopic pathology and microscopic pathology investigations were performed.

For F1 Cohort 2B, animals received no direct treatment, and no specific in-life investigations were performed. Animals were dispatched to necropsy at weaning for microscopic pathology investigations of the brain including brain morphometry.

For F1 Cohort 3, data was recorded on clinical signs, body weight, food consumption and sexual maturation. Spleen weight, macroscopic pathology and T-cell dependent antibody response investigations were performed.

**Results**

There was no evidence for silver in the blood from Control animals. Blood silver content increased with target dose however the increases were not dose proportionate and values in F1 PND 22 pups were significantly higher than adults. In general serum copper and selenium levels decreased as the target dose increased, the decreases in serum levels were not dose proportionate but tended to be greater for copper and for adult animals when compared with offspring on PND22. There were no statistically significant differences in thyroid stimulating hormone or thyroxine serum concentrations levels in any group or generation of males or females after dietary administration of silver Acetate at 40, 80 and 120 mg/kg/day when compared with Controls.

Overall modest effects were observed on the immunophenotyping parameters measured in Sprague Dawley rat spleen leukocytes as a result of the dietary administration of Silver Acetate, with a statistically significant increase ($p<0.05$) in the percentage of B cells in females at 40, 80 and 120 mg/kg/day however, the increase in the percentage of B cells in females did not translate into an increase in IgM production as no significant differences were found between the Control Group and the treated groups in the anti-keyhole limpet hemocyanin (KLH) IgM analyses (TDAR).

**F0 responses**

The mean achieved dose levels for the pre-pairing treatment period were within 10% of target dose levels of 40, 80 or 120 mg/kg/day. During gestation and lactation, the mean achieved dose levels for females were more variable, but all exceeded the target dose.

One male at 80 mg/kg/day and two males at 120 mg/kg/day died but the cause of death was undetermined. One female at 120 mg/kg/day was killed owing to a mammary lesion which was unrelated to treatment.

Body weight gain for males at 80 and 120 was significantly reduced at termination ($p<0.05$); female body weight was unaffected by treatment.

Intermittent, transient effects on food intake were observed for males at 40, 80 or 120 mg/kg/day and females at 120 mg/kg/day in Week 4 and during Days 4 to 13 of lactation.

The efficiency of food utilization for animals before pairing and for females during gestation was unaffected by treatment.

There was no effect of treatment on oestrous cycles, pre-coital interval, mating performance or fertility and the gestation index (number of litters born/number of mated females) was 100% in all groups. A marginal shift toward a longer gestation length was observed at 120 mg/kg/day.

Haematology at Week 10 for females and termination for males and females showed differences in erythrocyte count, mean cell haemoglobin and mean cell volume, red cell distribution width, mean cell haemoglobin, mean haemocrit, haemoglobin concentration, platelet count and white blood cell count with variable dose response and with males more affected than females. Females at 120 mg/kg/day showed high counts for eosinophils, monocytes and large unstained cells. All other parameters measured showed no effect of treatment.

Blood chemistry for females in Week 10 of treatment and for both sexes at termination showed, principally, high alkaline phosphatase activity, high plasma cholesterol and low potassium levels but differences were not always dose proportionate. There was a treatment related effect on testis weight and spermatid total millions, cauda epididymis sperm count and total millions following treatment with silver acetate at 120 mg/kg/day; however motile, progressive, motion and morphology were unaffected.

Body weight-relative heart weight was high for both males and females at all dose levels and all treated groups of males had low mean absolute pituitary weights and absolute/body weight-relative mean prostate weights. Low mean absolute testes weight was also observed at 80 and 120 mg/kg/day and high mean body weight-relative spleen weight was observed at 120 mg/kg/day. Females that received 120 mg/kg/day showed low absolute and body weight-relative mean thymus weight.

Most macroscopic findings at scheduled termination comprised abnormal colouration of tissues, including the gastrointestinal tract, kidneys, lacrimal glands, liver, harderian glands, mesenteric lymph nodes, pancreas, preputial/clitoral glands, salivary glands, thymus, thyroids, urinary bladder and uterus.

At histopathology, extracellular pigment was observed in various organs/tissues and was considered to represent deposition of test item at these sites. In general, pigment was more prominent in females, and there was not always an apparent dose response. Pigment was not associated with any inflammatory or degenerative changes.

Other histopathological findings included increased extramedullary haematopoiesis observed in the spleen of males at 80 or 120 mg/kg/day and epithelial degeneration of the glandular mucosa of the stomach in females that received 40, 80 or 120 mg/kg/day.

**F1 litter responses**

At 120 mg/kg/day was an increased incidence of offspring mortality and dark coloration; clinical condition of offspring at 40 or 80 mg/kg/day was considered unaffected by administration of silver acetate.

There was no effect of treatment on the number of implantation sites, mean litter size or sex ratio on postnatal Day 1.

At 120 mg/kg/day the live birth and viability indices were low when compared with Controls, resulting in a mean live litter size on Day 1 of 13.0 compared to 14.5 in Controls and 11.7 on Day 4 of compared to 14.4 in Controls; no differences were apparent at 40 or 80 mg/kg/day. Following litter standardization on Day 4 of age offspring survival at all dose levels was similar to Controls.

Mean body weight for male and female offspring at 120 mg/kg/day was low when compared with Controls on Day 1 of age and remained low until weaning. At 80 mg/kg/day mean offspring body weight on Day 1 was similar to Controls but lower than Controls on Days 14 and 21.

There was no effect on offspring weight or weight gain at 40 mg/kg/day. There was no effect of treatment on anogenital distance in male or female offspring and no effect on nipple count in males.

Macroscopic findings for offspring that died or were culled on Day 4 of age were predominantly absence of milk in the stomach and/or autolysis. There were no significant macroscopic findings in offspring terminated on Day 22 of age. At 80 or 120 mg/kg/day low mean absolute and body weight-relative thymus weights ($p<0.01$) were observed in both sexes, and females at 40 mg/kg/day showed low relative weights.

On PND22 mean absolute brain weight was low in females at 80 and 120 mg/kg/day.

**F1 responses**

The mean achieved dose levels for selected F1 animals were 36, 72 or 110 mg/kg/day for males and 40, 81 or 116 mg/kg/day for females and were no more than 10% below target dose levels of 40, 80 or 120 mg/kg /day.

Treatment at 120 mg/kg/day was associated with nine male and two female mortalities between Day 19 and Day 47 of the F1 generation. Of these seven decedents were subject to full microscopic examination which revealed five with brain lesions which were considered the major factor contributing to these deaths.

Following the high incidence of mortality at 120 mg/kg/day the high dose group was terminated prematurely at approximately 10 weeks of age.

Males at the target dose of 120 mg/kg/day showed a higher incidence of piloerection, hunched posture and abnormal gait; these signs were also observed in a few females at 120 mg/kg/day but at a low incidence.

At weaning on PND21 selected males and females receiving 80 or 120 mg/kg/day had low mean bodyweight and subsequent bodyweight gain up to PND25 was low at all target dose levels ($p<0.05/0.01$) with no clear dose response.

From Day 1 of the F1 generation (PND28±2) up to termination mean body weight, mean body weight gain and food consumption was low for males at all target dose levels; a dose response was apparent.

During Weeks 1-2 of the F1 generation treated females showed low body weight gain and food consumption. During Weeks 2-5 weight gain was high for treated females and from Week 5 body weight gain was similar to Controls. Periods of high food consumption were recorded at 80 and 120 mg/kg/day from Week 3-6; subsequent consumption for treated females was similar to Controls.

Overall, the efficiency of food utilization was unaffected by treatment for both males and females.

The mean age at completion of balano preputial separation was essentially similar across the groups but the mean bodyweight at completion was statistically significantly lower than Controls at all dose levels ($p<0.01$).

The mean age at completion of vaginal opening for females receiving 120 mg/kg/day was 1.2 days later than Controls ($p<0.01$) and the mean bodyweight at completion was statistically significantly lower than Controls at all dose levels ($p<0.05/0.01$).

The interval between vaginal opening and the first oestrus and oestrous cycles were unaffected by administration of silver acetate.

Hematology at scheduled termination (approximately 13 weeks of age) of males at 80 mg/kg/day and females at 40 or 80 mg/kg/day showed high mean erythrocyte counts. Males at 80 mg/kg/day also showed low mean cell haemoglobin, low mean cell volume and high red cell distribution width and females at 80 mg/kg/day showed high haemoglobin and high haematocrit. Conversely males and females at 120 mg/kg/day that were terminated prematurely at approximately ten weeks of age showed low erythrocyte counts, high mean cell haemoglobin and high mean cell haemoglobin concentration. In addition, females at 120 mg/kg/day had a low haematocrit, low red cell distribution width and high mean cell volume.

White blood cell parameters at either scheduled or premature termination were high for females that received silver acetate. Mean platelet counts were low for females at 80 or 120 mg/kg/day, prothrombin times were shorter in all treated groups of females and activated partial thromboplastin time was shorter for females at 120 mg/kg/day.

At premature termination blood chemistry for animals at 120 mg/kg/day principally showed high alkaline phosphatase activity, high alanine amino-transferase activity, high aspartate amino-transferase activity (females only), high cholesterol, high glucose, high urea (males), low creatinine, low albumin/A:G ratio, high potassium, low phosphorous levels and low sodium(females).

At scheduled termination of animals at 40 or 80 mg/kg/day blood chemistry investigations revealed high alkaline phosphatase activity (males), high alanine amino-transferase activity (males), high cholesterol and low creatinine.

Troponin investigations conducted on the F1 generation revealed higher levels in treated males, with mean levels at 120 mg/kg/day were slightly high but much lower than the mean values at 40 or 80 mg/kg/day (no historical control data were available for comparison). Cardiac troponin levels were unaffected in females at all dose levels.

Urinalysis investigations of the F1 generation revealed a low total protein output and protein concentration in males at 80 mg/kg/day (Urinalysis assessment was limited to Control, low and mid dose animals).
For auditory startle response the majority of latency to peak values for all groups of treated females were low compared with Controls and statistical significance was achieved for females at 120 mg/kg/day during trials 31-40. Group mean auditory startle latency to peak values for males at 120 mg/kg/day was high during trials 21-30.

The majority of group mean auditory startle peak amplitude values for males at all dose levels and females at 120 mg/kg/day were low when compared with Controls, with animals at 120 mg/kg/day showing the least habituation.

The majority of group mean high (rearing) and low (ambulatory) beam activity scores were low for males and females at 80 or 120 mg/kg/day.

In cage, in hand and arena observations showed a number of findings predominately at 120 mg/kg/day but also in a few animals at 80 mg/kg/day including, but not restricted to, abnormal motor movements, gait and activity.

Reactivity investigations showed several observations predominately at 120 mg/kg/day but also some were observed at 80 mg/kg day including reduced approach response, a weak startle response, failed pupil closure, high landing foot splay, reduced forelimb/hindlimb grip strength. In addition, females at all dose levels showed slightly low body temperatures.

Mean ovarian follicle and corpora lutea counts at 120 mg/kg/day were similar to Controls and considered unaffected by administration of Silver Acetate.

Only 12 F1 males were available for assessment of sperm parameters and these males were terminated approximately 3 weeks earlier than other groups. There was an effect on

cauda epididymal spermatid counts and an increased incidence of abnormal morphology (particularly decapitates, mainly in one animal) at 120 mg/kg/day.

For F1 Cohort 1A animals treatment related organ weight changes were noted in the heart in both sexes, the brain in males given 120 mg/kg/day, and the thymus in both sexes at 120 mg/kg/day. F1 Cohort 1B males also showed treatment related organ weight changes in the heart.

For F1 Cohort 2A males at 120 mg/kg/day had high body weight relative brain weight; this was not evident in Cohort 2B.

For F1 Cohort 3 higher than control mean absolute and body weight relative spleen weights were noted in both sexes at 120 mg/kg/day.

Treatment-related macroscopic findings in F1 Cohorts 1A, 1B, 2A and 3 were generally observed in both sexes across all dose groups without a significant dose response and were generally limited to abnormal coloration (dark) of affected organs/tissues. This was generally observed at a lower incidence and in fewer tissues compared to F0 generation animals. This abnormal coloration was no apparent in F1 Cohort 2B animals that were necropsied at weaning.

In the brain, dose related neuronal necrosis in the hippocampus, and neuronal/glial cell necrosis in the thalamus were observed in both sexes given 80 or 120 mg/kg/day. Intramyelinic oedema in the thalamus, caudate putamen and/or the corpus callosum was also observed in males given 80 or 120 mg/kg/day and in females given 120 mg/k/day.

Brain morphometry measurements revealed statistically significantly low hippocampus measurement for males that received 80 or 120 mg/kg/day.

**Conclusion**

Dietary administration of Silver Acetate at target 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 neurobehavior/sensory function at 80 or 120 mg/kg/day; motor activity for F1 males and females at 80 or 120 mg/kg/day; Sperm counts at 120 mg/kg/day for F0 males and all dose levels for F1 males; F1 sperm morphology at 120 mg/kg/day; Epithelial degeneration of the glandular mucosa of the stomach in F0 females at all dose levels; F1 histopathological findings of intramyelinic oedema and neuronal and/or glial cell necrosis at 80 or 120 mg/kg/day and F1 brain morphometry (low mean hippocampus) at 80 or 120 mg/kg/day. The minor reductions recorded in F0 and F1 male body weight gain up to 8% and 14% lower than Controls respectively were judged non adverse.

It was therefore concluded that the various no observed adverse effect levels (NOAELs) on this study were:

- Systemic toxicity in F0 adults: not established (due to degeneration in stomach mucosa in females at all doses).

- Systemic toxicity in F1 adults: 40 mg/kg/day (due to the following effects of treatment at 80 or 120 mg/kg/day: reduced activity and rearing of males and females in the arena, reduced reactivity, abnormal motor movement/gait, intramyelinic oedema and neuronal and/or glial cell necrosis and F1 brain morphometry (low mean hippocampus) – mean achieved doses of 36 mg/kg/day in males and 40 mg/kg/day in females.
- F0 mating performance and fertility: 120 mg/kg/day - mean achieved doses of 113 mg/kg/day for males and 127 mg/kg/day for females.

- F1 offspring survival and growth up to weaning: 80 mg/kg/day (due to reduced offspring survival and reduced growth at 120 mg/kg/day) – mean achieved doses of 100-107 mg/kg/day.

- Developmental neurotoxicity in selected F1 animals: 40 mg/kg/day (due to the following effects of treatment at 80 or 120 mg/kg/day: reduced activity and rearing of males and females in the arena, reduced reactivity, abnormal motor movement/gait, intramyelinic edema and neuronal and/or glial cell necrosis and F1 brain morphometry (low mean hippocampus) – mean achieved doses of 36 mg/kg/day in males and 40 mg/kg/day in females.

- Developmental immunotoxicity in selected F1 animals – 120 mg/kg/day – mean achieved doses of 110 mg/kg/day in males and 116 mg/kg/day in females.

**ANNEXES**

**Annex 1: Deviations from study plan of dose range finding study (Renaut et al., 2021)**

The following deviations from study plan occurred:

Day 25 Gestation Dispatch (Failed to Litter)
On Day 25 after mating after the 09:00 parturition check animal Group 2 female no. 121 and Group 3 female no 134 were due to be dispatched to necropsy as Failed to Litter as per study plan. These animals were not dispatched until Day 26 after mating in error. No adverse signs were documented against these animals throughout this period. Impact analysis: Both females were not pregnant, and no loss of data occurred; therefore, this study plan deviation had no impact on either animal welfare or the integrity of the study.

Dispatch
The selection file stated that for Group 1 litter no. 111, offspring M2 and F9 were to be retained on study as spare animals; however, these animals were despatched to necropsy on Day 21 of age in error.

Impact analysis: The study plan states that two male and two female animals per group will be selected as 'spare' animals, this deviation from study plan results in Control 'spare' animals being limited to one male and one female. This deviation from study plan had no impact on the integrity of this preliminary study as the 'spare' animals were not needed.

Visual Water Consumption
The Study Plan required qualitative (visual) assessment of water consumption. This was not scheduled on the Pristima data collection system and was not performed up to 28 July 2020 and commenced in Week 5 of study.

Impact analysis: Although formal observations/recordings were not performed the animals/cages were observed twice daily until Week 5 and no significant difference in water consumption was observed.

Liver weight not taken.
Contrary to study plan, the liver weight for Group 7 female 169 was not taken in error.

Impact analysis: The omission of a single liver weight in this preliminary study has no impact on study integrity.

These deviations were considered to have not affected the integrity or validity of the study.

## <u>Annex 2:</u> Deviations from study plan of EOGRTS study (Renaut et al., 2021)

| Category | Deviations |
|---|---|
| Animal Data Collection | It was a study plan requirement that on Day 22 a body weight and clinical sign was recorded. In error pup F8 from Dam 1F 224 did not have these recorded prior to dispatch.<br><br>This isolated omission has no impact on study integrity. |
| Animal Husbandry | It was a study plan requirement that the animals had access to the treated diet until the date of dispatch when it was replaced by pelleted diet. When the unselected pups from Dams 1F 213,214,220 3F 262,264 4F 285 were weaned from mum they were given pelleted diet in error. These animals were then dispatched on the 18 Jun.<br><br>Group 3 Litter 262 and 264 and Group 4 litter 285 underwent biosampling for Silver analysis on PND22; PI was informed of the error. This did not impact on study integrity. |
| Samples/Reagents/Test Materials - Handling | Contrary to protocol, the serum taken for Copper/Selenium analysis for the F0 females was placed into a -80°C freezer on the 25 June 2021. The error was noticed, and the samples transferred to -10 to -30°C storage on the 29 June 2021.<br><br>PI advised of deviation and from their perspective this had no impact on the integrity of the study samples. "Acceptable stability at -20°C infers stability at -80°C (Arrhenius equation). Furthermore, while we have not conducted previous studies for selenium, we have previously conducted numerous copper studies in which we have assessed stability in matrix at -80°C out as far as 1 year." |
| Sampling Error | Fecal samples for animals 4M 467 and 4F 768 were not taken from the rectum before it was immersed in neutral buffered formalin. After approximately 30 minutes the rectums were removed from the fixative, rinsed and then the fecal samples were taken.<br><br>This equates to 2/9 decedents; the majority of samples had been taken and were suitable for screening. |
| Incorrect/Missed Sample/Tissue Collection | Contrary to protocol F2A animal 4M 576 was only subject to a macro with abnormality's retained instead of a full list retention which was confirmed by Mark Blee who had contacted Ruth Renaut.<br><br>This was a full Cohort OECD443 and as such retention of abnormalities only for this decedent animals had no impact on the study integrity or validity. |
| Sample Processing | Contrary to protocol, no valid organ weight was taken for the prostate of 1M 483, as the value recorded was found to be erroneous at data check. The organ was re-weighed post-fixation.<br><br>This deviation for a Control male in Cohort 1B had no impact on study integrity. |
| Data Recording/Documentation Error | Contrary to protocol, no valid organ weight was taken for the left testis of animal 24 or the liver of animal 61, as the weights recorded were found to be erroneous at data check and were consequently excluded. |

| Category | Deviations |
|---|---|
| | These exclusions had no impact on study integrity. |
| AH - type 2, Sample Collection | It was a Study plan requirement that urine samples were obtained prior to termination. No sample was obtained for 3F 742 as the collection pot was positioned incorrectly under the funnel resulting in the urine missing the pot.<br><br>The lack of sample for one animal had no impact on the study integrity. |
| Sample Collection | Immuno spleen for animal 1M 418 placed into formalin in error, sample taken out straight away and rinsed with saline and placed into HBSS.<br><br>Analytical department informed, impact was not known until data analysis and data review was complete. |
| Missed/Extra Procedure | Contrary to protocol, no heart sections for transmission electron microscopy (TEM) were taken for animals 1F 711 +712.<br><br>The omission of two Control samples had no impact on study integrity. |

These study deviations neither affected the overall interpretation of study findings nor compromised the integrity of the study.

**Annex 3**

# Findings from an Extended One Generation Reproductive Toxicity Study on Silver Acetate: Perspectives on effect mechanisms and relevance to other forms of silver

Opinion prepared for the

European Precious Metals Federation aisbl

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The opinions and conclusions expressed herein originate from
Raffray Biosciences ltd, CM3 6NF Essex, UK, and do not represent the position of any other individual or organisation (unless otherwise stated).

**Executive Summary**
It is considered very probable that a copper deficiency state is the primary mechanism underlying a constellation of systemic and reproductive effects seen in an Extended One Generation Reproductive Toxicity Study (EOGRTS) in rats exposed to an ionic Ag reference (silver acetate). This conclusion is drawn from measurements of Cu status; similarities between the treatment-related effects and pathognomonic markers of copper deficiency; and the well-understood propensity of ionic Ag to perturb Cu homeostasis. At the dose levels associated with the main treatment-related findings in the EOGRTS, the deficiency state can be categorised as either moderately-severe or moderate in its degree. Neuropathological and neuromorphology changes which were evident in the brain of offspring are evaluated as being primarily due to indirect copper deficiency sequelae (rather than a direct neurotoxic effect of ionic Ag). Perspectives are offered herein on the predicted relative reproductive hazard of bulk elemental Ag (e.g. micron-sized powders or massive forms).

## 1) Introduction

Raffray Biosciences ltd have been asked to provide an expert opinion on certain findings arising from a recently completed Extended One Generation Reproductive Toxicity Study (EOGRTS) on an ionic silver compound (silver[I] acetate)[1]. In particular, as to whether a primary mechanism underlying the observed treatment-related effects could be a copper homeostasis shift in the $Ag^+$-treated animals. In addition, we have been requested to provide perspectives as to the significance of such a mechanism of action in the case of bulk elemental silver forms (e.g. micron-sized powder and massives) within the context of reproductive hazard potential.

Copper is an essential metal micronutrient. It is well established that significant Cu deficiency states (Cu-D) can produce a wide array of detrimental effects in mammals including adverse reproductive consequences, such as developmental disorders (US NRC, 1995; Delbeke, 2004; Halfdanarson et al., 2008; Uriu-Adams and Keen, 2005; Gambling and McArdle, 2004). This has been demonstrated for situations of both primary/direct Cu-D (i.e. low dietary intake) and secondary/indirect Cu-D (i.e. modulated by causes other than reduced dietary absorption of Cu – such as in vivo treatment with Cuchelators). Attempts have been made to develop categorisations for severity ranking of Cu-D in humans and laboratory animals (for example refer to Delbeke, 2004). Whilst quite arbitrary, these descriptive systems can be operationally useful in facilitating the grading of the deficiency state – for instance, as being either minimal, moderate, moderately-severe, or severe.

Evidence exists from experimental animal studies that treatment with ionic silver can deplete systemic copper levels (e.g. see Lison et al., 2021); mainly by causing structural deformation of the carrier protein ceruloplasmin, thus interfering with its capacity for copper-binding (Hirasawa et al., 1997; Sugawara and Sugawara, 2000; Ilyechova et al., 2014). However, Cu transport and extracellular Cu pools are not the only locus of action for $Ag^+$. The cellular transmembrane protein CTR1 is a transporter system with a key role in cellular Cu trafficking – regulating both intestinal and cellular uptake. Its action is essential to various biological processes including embryofetal development, and normal cardiac and liver function (Lee et al., 2001; Nose et al., 2006). $Ag^+$ is interchangeable with ionised Cu as a CTR1 substrate and therefore interferes with cellular Cu uptake (Lee et al., 2002; Zimnicka et al., 2007). The rate of Cu ion transport via the

[1] Renaut R et al. (2022) Silver Acetate: Extended One Generation Reproductive Toxicity Study in the Sprague Dawley Rat by Dietary Administration. Labcorp Early Development Laboratories Ltd. Labcorp Study Number 8437234 (draft audited report).

CTR1 system is comparatively slow (Ren et al., 2019) which represents a functional vulnerability if the local competing $Ag^+$ concentration is appreciable.

A linkage between indirect Cu-D induced by ionic Ag treatment and consequent developmental effects in rodents has previously been described (Shavlovski et al., 1995). However, before availability of the EPMF-sponsored EOGRTS (and an associated preliminary dose-range finding study[2]), a comprehensive interrelation of copper status with systemic and reproductive toxicity parameters in

ionic Ag-treated rats was a data gap. Commentary within this opinion also takes into account outcomes from the ancillary toxicokinetic and biomarker investigations, i.e. those embedded in the EOGRTS and preliminary studies which cover blood Ag and serum Cu measurements. It is noted that the potential mechanistic significance of indirect copper-deficiency caused by ionic silver has already been considered by The European Chemicals Agency (ECHA) Committee for Risk Assessment (RAC). For instance, in relation to CLH dossiers submitted for silver-containing substances[3, 4].

**2) Copper status of EOGRTS animals treated with ionic Ag**

The silver acetate (AgAc) dose levels and corresponding silver equivalent exposures achieved during the EOGRTS (Renaut et al., 2022) are summarised in Table 1. For F0 and F1 animals, actual exposures were generally at least 90% of the target; in some cases, exceeding the planned nominals by up to ~40% (e.g. for F0 dams during gestation and lactation).

TABLE 1: EOGRTS (AgAc) - nominal and achieved dose levels for F0 and F1 generation animals (a) F0 generation

| Treatment Group | Nominal (target) dose level | | Achieved dose level | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | mg AgAc/kg bw/d | mg Ag/kg bw/d♦ | ng AgAc/kg bw/d | | | | mg Ag/kg bw/d♦ | | | |
| | | | m | f (PM) | f (G) | f (L) | m | f (PM) | f (G) | f (L) |
| Low-dose (LD) | 40 | 26 | 38 | 42 | 52 | 56 | 25 | 27 | 34 | 36 |
| Mid-dose (MD) | 80 | 52 | 76 | 87 | 100 | 107 | 49 | 56 | 65 | 69 |
| High-dose (HD) | 120 | 78 | 113 | 127 | 152 | 149 | 73 | 82 | 98 | 96 |

(b) F1 generation

| Treatment Group | Nominal (target) dose level | | Achieved dose level | | | |
|---|---|---|---|---|---|---|
| | mg AgAc/kg bw/d | mg Ag/kg bw/d♦ | mg AgAc/kg bw/d | | mg Ag/kg bw/d♦ | |
| | | | m | f | m | f |
| Low-dose (LD) | 40 | 26 | 36 | 40 | 23 | 26 |

[2] Renaut R et al. (2021) Silver Acetate: Preliminary Reproductive Performance Study in the Sprague Dawley Rat by Dietary Administration. Labcorp Early Development Laboratories Ltd. Labcorp Study Number 8436495 (draft audited report).

[3] ECHA Committee for Risk Assessment (RAC) Opinion proposing harmonised classification and labelling at EU level of Silver zinc zeolite (Zeolite, LTA1 framework type, surface-modified with silver and zinc ions) EC Number: - CAS Number: 130328-20-0. CLH-O-0000001412-86-90/F. Adopted 4 December 2015.

[4] Proposal for Harmonised Classification and Labelling under Regulation (EC) No 1272/2008 (CLP Regulation), Annex VI, Part 2. Substance Name: Silver (CAS Number: 7440-22-4). Prepared by Swedish Chemicals Agency (KEMI), Version number: 3. Dated: September 2020.

| | | | | | | |
|---|---|---|---|---|---|---|
| Mid-dose (MD) | 80 | 52 | 72 | 81 | 47 | 52 |
| High-dose (HD) | 120 | 78 | 110 | 116 | 71 | 75 |

PM = pre-mating period G = gestation period L = lactation period

Values are mean intakes averaged over the specified period of exposure. ♦ Silver equivalent treatment level.

Section 3.2 of the EOGRTS report details dietary formulation procedures used to maintain correspondence with target dose levels.

AgAc treatment in a preliminary/dose-range finding study to the EOGRTS included two higher dose levels (160 and 320 mg AgAc/kg bw/d); achieved exposures for F0 male and female rats were not less than 95% of the target dose levels of 4, 40, 80, 160, 320 mg AgAc/kg bw/d (Renaut et al., 2021).

An evaluation of the copper status of F0 and F1 animals during the EOGRTS and preliminary study is possible via the serum Cu measurements obtained in these investigations. Concurrent determinations of total Ag in blood provide a toxicokinetic correlate. Table 2 summarises the available datasets from the EOGRTS for both parameters. It should be noted that interpretation of the PND 22 results is complicated by: (a) multiple statistical outlier values evident in the serum Cu for individual animals; (b) highly variable direct exposures to orally administered test articles, commonly existing at the point of weaning; and (c) the low bodyweight of pups at that stage, which can lead to transiently very high

(mg/kg bw) exposures in comparison to adults or juveniles. Table 2a incorporates this dataset after removal of outlier datapoints. In overview, the following conclusions may be drawn:-

i. For F0 animals, subject to achieved AgAc intakes of 38-152 mg AgAc/kg bw/d (25-98 mg Ag/kg bw/d), there was clear evidence of Cu level depression in all treated groups, for both genders. ii. A dose-response was apparent. The degree of effect was more prominent for males.

iii. The resultant Cu deficiency (Cu-D) state for these adult F0 animals is considered to be moderately-severe at the HD, i.e. a depression to about 20% of the control mean by Week 10 (i.e. just prior to mating). At the lowest treatment level (LD), i.e. achieved dose levels of 38-56 mg AgAc/kg bw/d, equivalent to 25-36 mg Ag/kg bw/d, Cu levels were decreased to 26-37% of the control for F0 males and 40-44% for females.

iv. Based on comparison of Week 10 and terminal (PND28) circulating Cu levels, there was no worsening in the degree of induced Cu-D state for F0 females, i.e. spanning a period which encompassed gestation and lactation for the dams[5].

v. A comparable treatment-related pattern of Cu level depression was evident for F1 animals. Based on relative decrements versus controls, it was not more severe in degree than that observed for the F0 generation (including for a timepoint where direct intake of the test article via the diet was occurring).

vi. In line with expected age-related differentials in the rat, the PND22 absolute values were lower than those evident at F1 termination, but were still sustained to 50%, or higher, of the control mean for the AgAc-exposed groups.

vii. In terms of gender sensitivity, based on terminal measurements the depression in Cu levels for F1 males (in Cohort 1A/1B) was typically more pronounced than observed for females – particularly at the LD and MD. Even at the LD, the group mean Cu levels for F1A and F1B males were only one-third of the respective control mean at this timepoint.

[5] The study design precluded the determination of the Cu status of the test subjects during these periods.

viii. By the point of termination, F1 animals exhibited a broadly similar degree of Cu depression (relative to Control) to that evident in F0 animals following 10 weeks of exposure.

ix. In the case of F0 and also F1A/B treated groups, serum Cu concentrations were inversely related to increasing blood Ag concentration, but the relationship was not a simple monotonic one when evaluated across all dose levels. Interpretation of the F1 PND22 blood Ag results, and their relationship to serum Cu levels, is complicated by indeterminate patterns of indirect and direct exposure to the test article at the point of weaning.

TABLE 2: EOGRTS (AgAc) – serum Cu and blood Ag levels for F0 and F1 generation animals

(a) Serum Cu

| Treatment Group | Serum Cu level (ng/mL) % control mean | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | | | | F1 | | | | | |
| | Males | | Females | | Males | | | Females | | |
| | Wk10 | Term. | Wk10 | Term. | PND22 | F1A | F1B | PND22 | F1A | F1B |
| Control | 1287 | 1515 | 1800 | 1840 | 666 | 1091 | 1211 | 720 | 1623 | 1578 |
| Low-dose (LD) | 470<br>37 | 390<br>26 | 719<br>40 | 811<br>44 | 438<br>66 | 368<br>34 | 398<br>33 | 458<br>64 | 869<br>54 | 1020<br>65 |
| Mid-dose (MD) | 316<br>25 | 255<br>17 | 544<br>30 | 562<br>31 | 337<br>51 | 245<br>22 | 254<br>21 | 363<br>50 | 576<br>35 | 650<br>41 |
| High-dose (HD) | 245<br>19 | 176<br>12 | 374<br>21 | 448<br>24 | 383<br>51 | 217<br>20 | 161<br>13 | 341<br>47 | 286<br>18 | 378<br>24 |

Values are group mean absolute serum Cu concentration. Shaded entries are percentage of corresponding control value.

F0: Wk10 = Timepoint during Week 10 of premating period. Term. = Terminal timepoint (F0 females terminated at PND 28).

F1: Terminal timepoint - LD/MD animal age ~13 weeks (Cohort 1A), 14 weeks (Cohort 1B); HD ~10 weeks (Cohort 1A/1B).

Formal statistical outliers in PND22 data series were excluded before further data analysis.

TABLE 2 (cont.)

(b) Ag in blood

| Treatment Group | Ag in blood (ng/mL) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | F0 | | | | F1 | | | | | |
| | Males | | Females | | Males | | | Females | | |
| | Wk10 | Term. | Wk10 | Term. | PND22 | F1A | F1B | PND22 | F1A | F1B |
| Low-dose (LD) | 223 | 215 | 314 | 162 | 871 | 248 | 239 | 919 | 246 | 189 |
| Mid-dose (MD) | 299 | 293 | 322 | 229 | 1279 | 272 | 226 | 1400 | 286 | 201 |
| High-dose (HD) | 350 | 322 | 439 | 258 | 1767 | 404 | 402 | 1982 | 502 | 360 |

Values are group means (reported as ng/mL total Ag; analysed in whole blood). All control group results <LLOQ.

F0: Wk10 = Timepoint during Week 10 of premating period. Term. = Terminal timepoint (F0 females terminated at PND 28).

F1: Terminal timepoint - LD/MD animal age ~13 weeks (Cohort 1A), 14 weeks (Cohort 1B); HD ~10 weeks (Cohort 1A/1B).

Serum Cu status measurements obtained during the preliminary/DRF study provide limited further insights at higher ionic Ag exposure levels, i.e. in respect of two higher dose levels at 160 and 320 mg AgAc/kg bw/d (nominal). These correspond to Ag equivalent doses of 103 and 207 mg/kg bw/d, respectively. For F0 females at d12 of the 4-week premating exposure period (which is predicted to be a point of steady state Ag toxicokinetics), the group mean Cu levels were depressed to circa 20% and 10% of the control, respectively. When F0 dams from the same groups were assessed at GD6, the corresponding values were 17% and 8% of control[6]. In terms of context, it should be noted that maternal circulating Cu levels normally increase during pregnancy, and also that GD1-7 represents the key temporal window of embryofetal susceptibility to Cu deprivation. It is likely that the clear perinatal embryofetal effects, e.g. decreased post-implantation survival, which were detected at these dose levels were associated with the significant Cu-D state induced in the dams.

**3) EOGRTS effects – mechanistic aspects**

A number of responses related to treatment were observed in F0 and F1 rats exposed to AgAc. The following interpretative commentary considers whether it is likely that an indirect Cu deficiency state (Cu-D) mediated by $Ag^+$ exposure could have operated as a causative mechanism of action.

**a) Haematology / Biochemistry changes**

For F0 animals, treatment-related shifts in RBC-related parameters – including erythrocyte count, mean cell haemoglobin and mean cell volume and red cell distribution width – were evident at Week 10 of the premating period and at termination. In concert with histopathology findings of extramedullary hematopoiesis in the spleen (HD and MD groups), and accounting for the degree of RBC parameter disturbance, these changes are indicative of a compensated non-severe anemia. The anemia was apparently microcytic in character with evidence of anisocytosis. Blood haemoglobin concentration was largely unaffected by treatment. In terms of gender susceptibility, male animals were typically more affected. Statistically significant differences were evident at all treatment levels including the LD. Broadly similar RBC alterations were also evident for F1 (Cohort 1A) at termination, although the effects were not as pronounced (particularly at the low dose), and HD comparisons are complicated by the premature termination of that group. In overall terms, these findings are congruent with other descriptions of low-grade anemia after exposure of rats or nonrodent species to ionic Ag compounds or $Ag^+$-releasers in repeat dose studies (Boudreau et al., 2016; ECHA, 2020; Lourens et al., 2021; Renaut et al., 2021).

It is well established that direct or indirect Cu-D causes anaemia in experimental animals and also in humans, which may variably be microcytic, normocytic or macrocytic in character[7] (Lahey et al.,
1952; Williams, 1983; US NRC, 1995; US NRC, 2000; Delbeke, 2004; Pyatskowit and Prohaska,

2008). Severe anemia associated with Cu-D arises only when Cu levels are very low. Most commentators consider that the locus of the effect involves bone marrow erythroid progenitor cells. Though for rodents, it remains controversial as to whether Cu-D-induced anemia primarily occurs due to secondary iron-deficiency disturbances (Pyatskowit and Prohaska, 2008). In conclusion, the constellation of RBC-related haematological changes observed in

[6] As appropriate, after statistical analysis these values exclude datapoints formally identified as significant outliers.

[7] The more long-standing the Cu-D the more likely the red cell indices will become hypochromic / microcytic.

EOGRTS F0 and F1 animals appear entirely consistent with causation via depression of circulating Cu levels.

Blood clinical biochemistry changes detected in AgAc-treated F0 and F1 animals included rises in alkaline phosphatase (ALP) activity and increased plasma total cholesterol levels[8]. Dose-response trends were typically evident for both parameters on all occasions, and for both generations and genders. The ALP elevations were not particularly marked even at the HD, and any parallel increases of transaminase enzymes were unremarkable in degree (viz. a limited effect principally constrained to the F1A HD). Comparable effects on ALP and cholesterol parameters have been observed during repeat dose toxicity studies on ionic Ag and nanosilver (e.g. Sprando, 2017; Boudreau et al., 2016; ECHA, 2020; Lourens et al., 2021; Renaut et al., 2021; Kim et al., 2010; Lee et al., 2013). As was the case for the EOGRTS on AgAc, significant histopathological changes in the liver were not evident in these investigations.

Rather limited elevations in liver enzyme markers, including ALP, have been described in clinical human Cu-D states, and also within experimental animal models of direct or indirect Cu-D (for example, see Yu et al., 2019; Cholewińska et al., 2018). However, ALP isoenzymes are also notably associated with several non-hepatic locations such as bone, connective tissue and the intestine. It is plausible that the detected ALP shift could be from non-hepatic origin, given that Cu-D has impacts on skeletal system, connective tissues and the gut (Medeiros, 2016; Gambling and McArdle, 2004; US NRC, 1995; US NRC, 2000). In respect of the cholesterol findings, rats and other species exhibiting Cu-D due to dietary insufficiency display characteristic elevations in total cholesterol and free cholesterol levels in plasma (Burkhead and Lutsenko, 2013; Allen and Klevay, 1980; Lei 1983; Lefevre et al., 1986; Uriu-Adams and Keen 2005); which to a varying extent can also be associated with shifts in certain lipoproteins. Such Cu-D induced hypercholesterolemia has been ascribed to alterations in hepatic lipid metabolism – possibly because of perturbations in glutathione levels (Kim et al., 1992).

**b) Organ weight changes**

Increased relative heart weight was a finding for EOGRTS F0 and F1 (Cohort 1A/1B) rats. The effect appeared to extend to all treatment levels including the LD (not consistently being statistically significant for F1 LD); though a clear dose-proportionality trend was not always evident. Apart from some limited cardiac findings for sporadic decedents in the HD, histopathological changes in the heart were absent. Cardiac immunohistochemistry related to von Willebrand factor (VWF) and Collagen IV was unremarkable. Increased blood concentrations of the cardiac marker Troponin I were detected for F1 males at all treatment levels (though not in the case of females), but the doseresponse pattern observed in F1 males appeared very unusual. Cardiac hypertrophy has not been regularly reported in previous toxicity studies in adult animals exposed to either ionic Ag or nanosilver, and there is a paucity of reliable data involving neonates. However, enlargement of the heart was described in offspring of rats which were administered silver zinc zeolite in a twogeneration reproductive toxicity study (ECHA, 2020), though the co-exposures to zinc and zeolite moieties complicate the interpretation of cause. In a study conducted by Charehsaz and co-workers (2016), the hearts of dams dosed with AgNP on GD 7-20 appeared slightly enlarged compared to the control, but no equivalent information was presented for pups.

From a plethora of studies in the literature, it is very well established that a cardinal effect associated with Cu-D is cardiac enlargement (Medeiros, 2017). Many of these reports involve confirmatory work in rodent models (for instance, Prohaska and Heller 1982; Olivetti et al.,

[8] Investigations performed at Week 10 (F0) and at termination (F0 / F1).

1992). It has been proposed that this form of cardiac hypertrophy is not secondary to coincident anemia (Medeiros et al., 1991;

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Medeiros, 2017). It has been shown that even quite moderate Cu-D (via dietary restriction) is capable of inducing cardiac hypertrophy in male rats (Li et al., 2005). When considering the findings for F0 and F1 AgAc-treated animals in the EOGRTS, it is plausible that the limited degree of cardiac enlargement observed is linked to the depression of circulating Cu levels.

Reduced thymus weights were recorded for EOGRTS unselected F1 pups from all treated groups on d22. A dose-response was evident, although the difference at the LD was of borderline statistical significance (only the relative organ weight value for LD females). No similar effect was apparent on termination of F1A and F1B (i.e. at 10-14 weeks), and no treatment-related histopathological changes in the thymus were noted. Furthermore, adverse effects were absent in the F1 DIT cohort. In the parental generation, statistically significant decreases in thymic weight occurred in F0 HD females, but not males. Hence, the detected effect on F1 thymic weight was only transiently evident (at d22), and was not linked to later adverse structural or functional changes. Nevertheless, given that the first 3-4 weeks of development represents a known susceptibility window in thymic ontogeny, it is possible that the change was treatment-related. Cu-D has been associated with thymic involution in offspring, particularly when the maternal animal deficiency state is pronounced or chronic in nature (Prohaska et al., 1983) or in the case of neonates fed a Cu-depleted diet (Koller et al., 1987).

**c) Perinatal effects**

In the EOGRTS, the LOAEL for litter responses was considered to be 120 mg AgAc/kg bw/day (nominal), corresponding to exposure of the dams during gestation to 152 mg AgAc/kg bw/day (98 mg Ag/kg bw/day). This LOAEL placement was based on reductions in live birth and viability indices. HD group pups had lower bodyweight at timepoints from PND1 to PND21. No similar differences were evident at 40 or 80 mg AgAc/kg/day. The study design precluded determination of Cu levels in the dams at parturition, but via an interpolation from Week 10 and terminal values, the HD serum Cu levels may have been about 20-25% of control values.

When higher dose levels (160 and 320 mg AgAc/kg bw/d [nominals]) were evaluated in a preliminary study, more pronounced perinatal effects indicative of clear embryofetal toxicity were evident (Renaut et al., 2021). Most notably at 320 mg AgAc/kg bw/d (equivalent to 207 mg Ag/kg bw/d), where the post-implantation survival index was only 54.1%, and the live birth index was 15.3%. It was also found that pup bodyweight was significantly reduced. Examination of decedent offspring revealed no macroscopically observable abnormalities. These preliminary study findings correlate with a more severe Cu-D in the dams than attained in the EOGRTS (refer to Section 2).

The above now provides better context to a mechanistic investigation performed in rats by Shavlovski et al. (1995). In this work, silver chloride (estimated as 50 mg/animal; i.e. ~250 mg AgCl/kg bw/d, or 188 mg Ag/kg bw/d) was administered in the diet to female rats. Little information on achieved intake was detailed in the publication: actual ionic Ag exposure may have differed substantially from this estimate. Exposures were either during days 7-15 of gestation, or else throughout the entirety of gestation (GD1-20). AgCl administration during only the period of organogenesis did not affect the development of embryos, nor did it result in pre-implantation or post-implantation losses. In contrast, treatment throughout gestation resulted in a marked incidence of post-implantation losses; the number of live fetuses was

decreased by 81%; and the delivered pups exhibited reduced birth weights. Some pups had external and visceral abnormalities; and premature pup deaths occurred. It was reported that Cu levels were undetectable in placenta, embryonic tissues and maternal serum; with circulating ceruloplasmin ferroxidase activity being ablated. Based on such indicators, this points to the development of a very severe Cu-D state. From the above findings, and ancillary Cu-chelator experiments, the investigators concluded that GD1-7 represents a key embryofetal susceptibility window in relation to Cu deprivation.

It is notable that in respect of low-end dose-response for littering parameters, findings equivalent to those described by Sprando and co-workers (2017) in a rat one generation reproductive toxicity study were not replicated during the EOGRTS with AgAc (given that 40 mg AgAc/kg bw/d was established as a clear NOAEL in the EOGRTS).

**d) DNT findings (including neuropathology and CNS morphometry)**

Neurohistopathological abnormalities in the EOGRTS F1 generation were reported as intramyelinic edema and neuronal and/or glial cell necrosis at HD and MD (see Table 3). In comparison to the control, a reduced mean hippocampus measurement as assessed by brain morphometry was also apparent for F1 HD and MD male animals (Table 3). The degree of myelinopathy and neuronal/glial cell necrosis was not marked–being graded as minimal to slight. The neuropathology and morphometric findings were present in animals terminated at either 13 or 10 weeks of age (Cohort 1A) or PND 75 (Cohort 2A), but not in PND 22 pups (Cohort 2B), suggesting that onset was delayed.

A characteristically similar pattern of CNS changes has been elaborated in rodent species exhibiting Cu-D (Table 3; Hunt and Idso, 1995; Carlton and Kelly, 1997; Zimmerman et al., 1976; Dipaolo et al., 1974; Zucconi et al., 2007; Uriu-Adams et al., 2010; Zatta and Frank, 2006; Delbeke, 2004; Hurley and Keen, 1979). In these rodent models of Cu-D, including investigations on developing animals, the pattern and severity of brain neuropathology change (and morphometric change in certain brain regions) is quite consistent. It correlates with degree of Cu depletion state in affected animals – typically being identifiable in situations of severe, or at least moderate Cu deficiency. Overall, a high degree of correspondence exists between the EOGRTS F1 findings and the pathognomonic CNS changes of moderate Cu-D. Although glial cell activation is a common observation in Cu-D, it was not reported in the EOGRTS neuropathology outcomes; but this phenomenon can be subtle and difficult to discriminate. Synaptogenic abnormalities, e.g. within the hippocampus, are another frequent finding in the brains of animals affected by Cu-D; though visualisation is dependent on use of ultrastructural techniques not routinely applied in a standard EOGRTS design.

TABLE 3: Neuropathology and other CNS effects – comparison of findings (EOGRTS / Copper deficiency states / Other in vivo studies on ionic silver or nanosilver)

| | EOGRTS | Cu Deficiency (Cu-D) | Ionic Ag / AgNP |
|---|---|---|---|
| | Neuropathology and other CNS findings detected in F1 generation | CNS neuropathology pathognomonic for Cu-D♦ | Neuropathology associated with $Ag^+$ or AgNP exposure‡ |
| Myelinopathy | Intramyelinic edema (thalamus, caudate putamen and/or corpus callosum)<br>Cohort 1A: MD / HD<br>Cohort 2A: HD | Myelinopathies; including hypomyelination and vacuolation (brain, e.g. cerebellum; spinal cord) | Myelinopathy has been previously described (but the evidence is not robustly established) |

| | | | |
|---|---|---|---|
| Cell loss / death | Neuronal necrosis (hippocampus)<br>Neuronal/glial cell necrosis (thalamus)<br>Cohort 1A: MD / HD<br>Cohort 2A: HD | Neuronal cell death (multiple regions: including hippocampus, thalamus, cerebral cortex, corpus striatum) | Neuronal cell death (especially hippocampus) |
| Glial cell changes | Glial cell death (see above)<br>Glial cell activation (e.g. of microglia) was not reported | Activation of microglia and astrocytes (cerebral cortex, thalamus, hippocampus and cerebellum) | Glial cell activation; microglia and astrocytes (hippocampus) |
| Regional CNS morphometric effects | Size reduction in hippocampus<br>Cohort 2A: MD / HD (males) | Size reduction in hippocampus/ dentate gyrus; cerebellar Purkinje cell layer irregularities | Size reduction in hippocampus; cerebellar Purkinje cell layer irregularities |

♦ Emphasis on investigations covering developing animals or neonates (mainly rodent models).

♮ Based on previous publications covering rodents and other species, i.e. mainly non-TG research investigations in developing/neonatal animals (in some cases the reports relate to adults).

Note: No adverse CNS histopathology findings were reported for the EOGRTS F0 generation.

Whilst distinct CNS neuropathological change after in vivo exposure of adult animals to ionic Ag or nanosilver has only occasionally been reported, there are descriptions of effects in developing animals or neonates (Rungby et al., 1987; Skalska et al., 2015; Liu et al., 2012; Patchin et al., 2016; Tang et al., 2009; Charehsaz et al., 2016). Hippocampal changes including cell death and glial cell activation do feature in certain of these literature reports (Table 3) – again showing overlapping features with abnormalities associated with Cu-D states in rodents and other species.

From the neurological function assessments of EOGRTS F1 animals, deficits were apparent in auditory startle response, certain functional observational battery parameters and neuromotor activity (e.g. abnormal motor movements, gait and activity modes). These were clearly evident at the HD but in several cases were detected in MD rats (e.g. weakened startle response and reduced reactivity, or decreased activity levels alongside lowered beam break scores). Several of these effects are fully congruent with functional neurological changes described in rodent models of Cu-D. Associated neurological deficits in Cu-D include disordered neuromotor control; reduced activity; diminished avoidance and startle responses; and disturbance of complex behaviours and cognitive ability (Prohaska, 2000; Uriu-Adams and Keen, 2005; Delbeke, 2004; Penland and Prohaska, 2004; Prohaska and Hoffman, 1996; Lyons and Prohaska, 2010). These in turn have been connected to the impact of Cu depletion on functionally-linked brain regions such as the cerebellum, thalamus and hippocampus. Brain neurochemistry may also be affected, with the offspring of Cu depleted dams exhibiting alterations in neurotransmitter levels (Uriu-Adams et al., 2010; Nelson and Prohaska, 2009). In rodent models (and some other species), moderate perinatal Cu-D is sufficient to begin to induce neurological effects. The relative susceptibility of the brain may relate to the elevation in brain Cu content occurring in the developing foetus (McArdle, 1995), which is linked to support of normal brain development, e.g. for key cuproenzyme requirements.

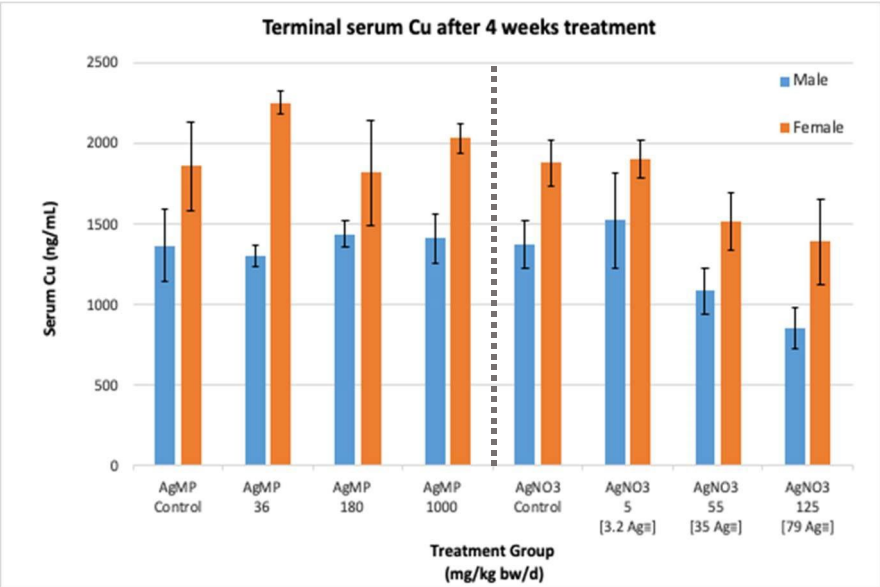
**4) Perspectives on bulk elemental Ag forms (e.g. micron-sized powders and massives)**

When considering the latest reproductive toxicity studies on AgAc, together with other toxicity investigations (e.g. on silver containing active substances), it is clear that rodents exposed via the oral route to ionic Ag compounds or $Ag^+$-releasers (ECHA, 2020) may develop Cu depletion states. As previously mentioned, the underlying mechanism is thought to involve Ag ions binding to, and interfering with, the key copper transporter protein ceruloplasmin. However, no comparable in vivo toxicity data exist in the specific case of bulk elemental Ag (e.g. micron-size or larger powders, or massive forms).

At dose levels relevant to CLH classification, it has recently been found that a type of bulk Ag (viz. a sub-micron sized powder [AgMP]) displays a quantitatively very different toxicokinetic character to that of either ionic Ag or nanosilver (Charlton et al., 2021; Melvin et al., 2021). In particular, considerably lower systemic and tissue-level exposures to bioavailable Ag were evident for AgMP versus either ionic or nanosilver references. In some instances, the internal exposure metric differences spanned several orders of magnitude. From in chemico dissolution studies it is also known that, in mass-normalised terms, bulk silver liberates orders of magnitude less $Ag^+$ than more bioavailable ionic or nanosilver forms. This difference is pertinent to their respective potentials for interference with Cu transporters in vivo, including ceruloplasmin.

The copper status of rats exposed to AgMP has been evaluated and compared to results for an ionic Ag reference (Melvin et al., 2021). The key findings from these experiments are summarised in Figure 1. For AgMP, there was no indication of any depletion in circulating Cu levels after 28 days treatment via the oral route, even with treatment corresponding to a limit dose (1000 mg/kg bw/d). This equates to a Ag equivalent dose level which was 12 times higher than that employed as the $AgNO_3$ high dose comparator. In contrast, a depression in circulating Cu levels was apparent from a quite low exposure in the case of the ionic reference compound (being evident from 35 mg Ag/kg bw/d) – refer to Figure 1.

FIGURE 1: Differences in serum Cu status of rats orally exposed to bulk Ag versus ionic Ag




| Dose level (mg/kg bw/d) | Serum Cu conc % of Control mean | |
|---|---|---|
| | Male | Female |
| AgMP 36 | 95 | 121 |
| AgMP 180 | 105 | 98 |
| AgMP 1000 | 103 | 109 |
| $AgNO_3$ 5 | 111 | 101 |
| $AgNO_3$ 55 | 79 | 81 |
| $AgNO_3$ 125 | 62 | 74 |

Animals were treated with either AgMP or $AgNO_3$ p.o. (daily by gavage) for 4 weeks.

Values are group mean serum Cu concentrations (with SD also shown).

Corresponding Ag equivalent treatment levels [Ag≡] are indicated in the case of animals which received $AgNO_3$.

Both the EOGRTS and this work were conducted on ionic Ag compounds recognised as possessing the highest relative bioavailability of the simple salts of silver (viz. AgAc and $AgNO_3$). In terms of relating the AgMP experimental observations to the latest reproductive toxicity studies on AgAc: it is acknowledged that the work on AgMP involved only adult animals; treated by gavage rather than dietary administration; over a relatively short period of 4 weeks. In mitigation of the last limitation, evaluation of serum Cu in the EOGRTS F0 animals showed that the degree of Ag-induced Cu depression was consistent irrespective of the duration of treatment. It has separately been determined that the degree of Cu-D in adult rats remained quite constant over the course of 4 to 10 weeks treatment in a repeat dose study with AgAc via the dietary route (Lison et al., 2021). In terms of Ag toxicokinetics, it is justifiable to relate the EOGRTS to the shorter duration study reported by Melvin et al., 2021; given that several experiments in rat models indicate the attainment of steady state systemic exposure after repeat oral dosing for a period of about two weeks.

High confidence comparisons between the Ag toxicokinetics and Cu datasets in the respective EOGRTS and TK studies for AgMP and AgAc are complicated by several factors. However, based on correlations of systemic exposure and peak exposure with circulating Cu status, there are grounds to doubt whether it would be possible to attain a condition of moderate or greater Cu depletion in the case of orally administered bulk Ag (AgMP)[9]. In particular, at AgMP dose levels of relevance to hazard classification.

### 5) Concluding remarks

In relation to the outcomes of an Extended One Generation Reproductive Toxicity Study (EOGRTS) on the ionic silver compound silver acetate (AgAc)[10] the following interpretative comments are offered:- a) Several of the treatment-related effects which occurred in F0 and F1 generation animals have close correspondence with archetypic changes present in direct and indirect copper deficiency states in experimental animals (and in humans). These included the findings in the EOGRTS of:

(i) haematological and biochemical shifts [mainly anemia together with elevated plasma alkaline phosphatase activity and hypercholesterolemia]; (ii) organ weight changes indicative of cardiac hypertrophy and thymic involution; (iii) changes in littering indices [reductions in live birth rate and pup viability]; (iv) pup growth rate depression, and development of poor condition or failure to thrive; (v) brain myelinopathy and cell loss, with diminution of the size of the hippocampus; and (vi) deficits in motor function and neurobehavioural parameters.

b) Such effects in F0 animals and their offspring were most evident at the HD, where the achieved dose levels in F0 dams during premating to gestation ranged from 127-152 mg AgAc/kg bw/d (equivalent to 83-98 mg Ag/kg bw/d). Estimated circulating Cu levels in these animals were reduced to circa 20-25% of the control, i.e. corresponding to a moderately-severe copper deficiency state. Littering parameters or pup growth were not adversely affected at the MD (where achieved intakes in F0 females during premating to gestation were 87-100 mg AgAc/kg bw/d; 56-65 mg Ag/kg bw/d). However, certain other treatment effects which were qualitatively similar to those detected in the HD were present, in turn being associated with moderate Cu-D in F0 females (~30% of control levels).

[9] EPMF have stated that the form/size of bulk silver (AgMP) which was tested is considered to represent a worst-case type of silver powder in terms of its expected bioavailability.

[10] Renaut R et al. (2022) Silver Acetate: Extended One Generation Reproductive Toxicity Study in the Sprague Dawley Rat by Dietary Administration. Labcorp Early Development Laboratories Ltd. Labcorp Study Number 8437234 (draft audited report).

c) Very marked differences in gender response patterns in the EOGRTS F1 generation were not obvious. However, higher susceptibility was apparent for F1 males for certain effects (e.g. sporadic death, loss of condition, and hippocampal size change), even though the attained blood Ag exposures were actually lower than those in females. As discussed in Section 2, serum Cu levels in F1 (1A/1B) males were consistently more depressed than in females. In rat models of Cu-D, male animals have often been shown to be more susceptible in terms of the severity of adverse effects and rapidity of onset of the syndromic changes (US NRC, 1995).

d) In mammalian Cu-D states, the tissues with greater susceptibility to adverse effects from disturbed Cu homeostasis include those with higher energetics demand or higher cuproenzyme support needs, e.g. the developing conceptus, CNS/brain, heart, liver, kidney, and skeletal muscles (listed in approximate rank sensitivity). The EOGRTS findings do align with this sensitivity pattern.

e) Cu depletion thresholds for major developmental effects are currently not well elucidated across various species, including humans. Overt developmental responses have been shown in the literature to be more commonly associated with at least moderately-severe deficiency states. In rat models, moderate copper deficiency, resulting in a 30% and 68% fall in maternal and neonatal copper levels respectively, has little effect on the number of viable pups born or on neonatal weight (Gambling et al., 2011). In a direct Cu-D study in rats, depression of plasma Cu to ~17% of the control value did not have marked effects on pregnancy outcomes or foetal viability (Masters at al., 1983). When taking into account the dose-response from a preliminary reproductive toxicity study on AgAc plus the EOGRTS (see Section 2), perinatal littering parameters are seriously affected when circulating Cu in dams is depressed to about 10% of normal, though some effect becomes discernible at 20% of the control value. If maternal Cu levels are reduced to undetectable levels, along with ablation of normal ceruloplasmin structure and function, then severe embryofetal lethality and developmental abnormalities occur in rodents (Hurley and Keen, 1979; Oster and Salgo, 1977; Shavlovski et al., 1995).

f) As discussed in Section 3/Table 3, the neuropathology and neuromorphometric changes in the brain of EOGRTS F1 animals do closely correspond with those typically exhibited in the CNS of developing animals affected by direct or indirect Cu-D. The changes observed were graded by the study pathologist as 'minimal' to 'slight', and collectively they correspond to those predicted for situations of less than severe Cu-D in rodents. These occurred in tandem with functional neurological deficits which were again archetypic of those associated with Cu-D. From a mechanistic and susceptibility perspective, it may be relevant that: firstly, levels of key Cu transporter CTR1, which is vulnerable to interference from $Ag^+$, are highly expressed at periphery of the CNS (Zheng, 2001); and secondly, developing animals have particularly high CNS Cu requirements (McArdle, 1995).

g) In considering the totality of evidence available for $Ag^+$-releasers (such as ionic Ag compounds and nanosilver), it is considered less likely that silver causes direct CNS neurotoxicity. Multiple oral route toxicokinetic studies in rodents have shown that the brain is not a major site of Ag distribution in either adult rodents or those exposed in utero (e.g. Charlton et al., 2021; Melvin et al., 2021; Charehsaz et al., 2016). In terms of brain parenchymal exposure, several investigations (typically based on autometallography as a technique, e.g. Rungby and Danscher 1983a, 1983b; and recent unpublished work by Lison and colleagues), demonstrate that much of the identifiable Ag depots in adults or progeny are actually associated with the CNS endothelial sub-compartment, or are deposited in basal

lamina of the choroid plexus. In turn, such observations suggest the existence of an effective blood-brain barrier (BBB) which limits ionic silver ingress. However, it is acknowledged that the embryofetal and neonatal BBB can be more permeable than in adults, and this is not a fully explored possibility in the case of Ag. In the event that $Ag^+$ does penetrate the BBB, e.g. by rate-limited active uptake, it will be quickly sequestered into ultralow solubility selenide and sulphide complexes. These are stable species considered to be of negligible toxicological significance.

h) As discussed in Section 3 regarding Cu-D linkages, good correspondence exists between the constellation of effects detected in the EOGRTS on AgAc and those found in other repeat dose toxicity and reproductive toxicity studies with $Ag^+$-donor substances (encompassing ionic Ag salts as well as other $Ag^+$-releasers, e.g. SCAS and nanosilver). This includes reasonable alignment with the outcomes of a two-generation reproductive toxicity study in rats performed on silver zinc zeolite (SZZ). Therein the key observations in offspring were elevated fetal/pup mortality, retarded growth and thymic involution (ECHA, 2015). The dietary Ag equivalent exposures associated with these effects were appreciably lower than those in the AgAc EOGRTS; being about 10 to 20 mg Ag/kg bw/d. Previous reviewers of the study have cautioned of interpretative difficulties due to the coexistence of zinc and zeolite–as well as silver–within SZZ. If it is valid that a Cu-D mechanism is central to the biological effect of Ag in vivo, this suggests a reason for why toxicity potentiation by Zn in SZZ may be an important consideration. It is very well established that Zn inhibits the intestinal absorption of Cu; and furthermore, a key study has found that $Zn^{2+}$-binding to the intracellular Cu transporter CTR1 is able to negatively regulate its Cu transport activity (Ren et al., 2019). It is also plausible that additive interference with Cu uptake from the GI tract occurs, since the Type A zeolite can directly adsorb Cu ions (Majdan et al., 2003).

## Abbreviations

| | |
|---|---|
| AgAc | Silver(I) acetate |
| AgNP | Silver nanoparticle(s) |
| ALP | Alkaline phosphatase |
| BBB | Blood-brain barrier |
| Cu-D | Copper deficiency |
| d | Day |
| CTR1 | High-affinity copper uptake protein 1 |
| DIT | Developmental immunotoxicity |
| DNT | Developmental neurotoxicity |
| DRF | Dose-range finding |
| GD | Gestational day |
| LD / MD /HD | Low-Dose / Mid-Dose / High-Dose (treatment group) |
| PND | Post natal day |
| RBC | Red blood cell |
| SCAS | Silver-containing active substance |
| SZZ | Silver Zinc Zeolite (a SCAS) |
| TG | Test guideline (conformant) |
| TK | Toxicokinetic |

## References


Allen KGD, Klevay LM (1980) Hyperlipoproteinemia in rats due to copper deficiency. Nutr. Rep. Int. 22: 295-299.

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.

Burkhead J, Lutsenko S (2013) The role of copper as a modifier of lipid metabolism. Lipid Metabolism. 39-61. doi: 10.5772/51819.

Carlton, WW, Kelly WA (1997) Neural lesions in the offspring of female rats fed a copper-deficient diet. J. Nutr. 97:4252.

Charehsaz M, Hougaard KS, Sipahi H, Ekici AI, Kaspar Ç, Culha M, Bucurgat ÜÜ, Aydin A (2016) Effects of developmental exposure to silver in ionic and nanoparticle form: A study in rats. Daru. 24: 24. doi: 10.1186/s40199-0160162-9.

Charlton A et al. (2021) Silver Nitrate, Micron-sized Silver and Nanoparticulate Silver: A Comparative Toxicokinetic Study in Rats by Repeat Administration. Labcorp Study Number: 8469368.

Cholewińska E, Fotschki B, Juśkiewicz J, Rusinek-Prystupa E, Ognik. K (2018). The effect of copper level in the diet on the distribution, and biological and immunological responses in a rat model. Journal of Animal and Feed Sciences. 27: 349-360.

Delbeke K (2004) Voluntary risk assessment of Copper, Copper II Sulphate Pentahydrate, Copper(I)Oxide, Copper(Ii)Oxide, Dicopper Chloride Trihydroxide.. APPENDIX HH-A Copper Deficiency. ECHA Voluntary risk assessment reports; https://echa.europa.eu/copper-voluntary-risk-assessment-reports

Dipaolo RV, Kanfer JN, Newberne PM (1974) Copper deficiency and the central nervous system: myelination in the rat: morphological and biochemical studies. J Neuropathol Exp Neurol. 33: 226-236.

ECHA Committee for Risk Assessment [RAC] (2015) Opinion proposing harmonised classification and labelling at EU level of Silver zinc zeolite (Zeolite, LTA1 framework type, surface-modified with silver and zinc ions) EC Number: - CAS Number: 130328-20-0. CLH-O-0000001412-86-90/F.

ECHA (2020) Proposal for Harmonised Classification and Labelling under Regulation (EC) No 1272/2008 (CLP Regulation), Annex VI, Part 2. Substance Name: Silver (CAS Number: 7440-22-4). Prepared by Swedish Chemicals Agency (KEMI), Version number: 3. Dated: September 2020. Released by ECHA for public consultation on 19 October 2020.

Gambling L, McArdle HJ (2004) Iron, copper and fetal development. Proc Nutr Soc. 63: 553-562.

Gambling L, Kennedy C, McArdle HJ (2011) Iron and copper in fetal development. Semin Cell Dev Biol. 22: 637-44.

Halfdanarson TR, Kumar N, Li CY, Phyliky RL, Hogan WJ (2008) Hematological manifestations of copper deficiency: a retrospective review. Eur J Haematol. 80: 523–531

Hirasawa F, Kawarada Y, Sato M, Suzuki S, Terada K, Miura N, Fujii M, Kato K, Takizawa Y, Sugiyama T (1997) The effect of silver administration on the biosynthesis and the molecular properties of rat ceruloplasmin. Biochim Biophys Acta 1336:195-201.

Hunt CD, Idso JP (1995) Moderate copper deprivation during gestation and lactation affects dentate gyrus and hippocampal maturation in immature male rats. J Nutr. 125: 2700-2710.

Hurley, LS, Keen CL (1979) Teratogenic effects of copper. pp. 33–56 in Copper in the Environment. Part II: Health Effects, J.O. Nriagu, ed. New York: John Wiley & Sons.

Ilyechova EY, Saveliev AN, Skvortsov AN, Babich PS, Zatulovskaia YA, Pliss MG, Korzhevskii DE, Tsymbalenko NV, Puchkova LV (2014) The effects of silver ions on copper metabolism in rats. Metallomics 6: 1970-1987.

Kim S, Chao PY, Allen KG (1992) Inhibition of elevated hepatic glutathione abolishes copper deficiency cholesterolemia. FASEB J 6: 2467–2471.

Kim YS, Song MY, Park JD, Song KS, Ryu HR, Chung YH, Chang HK, Lee JH, Oh KH, Kelman BJ, Hwang IK, Yu IJ (2010) Subchronic oral toxicity of silver nanoparticles. Part Fibre Toxicol. 7: 20. doi: 10.1186/1743-8977-7-20.

Koller LD, Mulhern SA, Frankel NC, Steven MG, Williams JR (1987) Immune dysfunction in rats fed a diet deficient in copper. Am J Clin Nutr. 45: 997-1006.

Lahey ME, Gubler CJ, Chase MS, Cartwright GE, Wintrobe MM (1952) Studies on copper metabolism. II Hematologic manifestations of copper deficiency in swine. Blood 7: 1053–1074.

Lee J, Prohaska JR, Thiele, DJ (2001) Essential role for mammalian copper transporter Ctr1 in copper homeostasis and embryonic development. Proc Natl. Acad. Sci. 98: 6842–6847.

Lee J, Pena MM, Nose Y, Thiele D (2002) Biochemical characterization of the human copper transporter Ctr1. J Biol Chem 277: 4380–4387.

Lee JH, Kim YS, Song KS, Ryu HR, Sung JH, Park JD, Park HM, Song NW, Shin BS, Marshak D, Ahn K, Lee JE, Yu IJ (2013). Biopersistence of silver nanoparticles in tissues from Sprague-Dawley rats. Part Fibre Toxicol. 10: 36. doi: 10.1186/1743-8977-10-36.

Lefevre M, Keen CL, Lönnerdal B, Hurley LS, Schneeman BO (1986) Copper deficiency-induced hypercholesterolemia:
effects on HDL subfractions and hepatic lipoprotein receptor activity in the rat. J Nutr. 116: 1735-1746.

Lei KY (1983) Alterations in plasma lipid, lipoprotein and apolipoprotein concentrations in copper-deficient rats. J. Nutr. 113: 2178-2183.

Lison D, Ambroise J, Leinardi R, Ibouraadaten S, Yakoub Y, Deumer G, Haufroid V, Paquot A, Muccioli GG, van den Brûle S (2021) Systemic effects and impact on the gut microbiota upon subacute oral exposure to silver acetate in rats. Arch Toxicol. 95:1251-1266.

Li Y, Wang L, Schuschke DA, Zhou Z, Saari JT, Kang YJ (2005) Marginal dietary copper restriction induces cardiomyopathy in rats. J Nutr. 135: 2130-2136.

Liu Y, Guan W, Ren G, Yang Z (2012) The possible mechanism of silver nanoparticle impact on hippocampal synaptic plasticity and spatial cognition in rats. Toxicol Lett. 209: 227-231.

Lourens NJJ et al. (2021) A 90-Day Study of Silver Acetate by Dietary Administration in Wistar Han Rats. Charles River Laboratories Den Bosch BV (The Netherlands). Test Facility Study Number 20274170 (draft unaudited report).

Lyons JA, Prohaska JR (2010) Perinatal copper deficiency alters rat cerebellar purkinje cell size and distribution. Cerebellum. 9: 136-144.

Majdan M, Pikus S, Kowalska-Ternes M, Głdysz-Płaska A, Staszczuk P, Fuks L, Skrzypek H (2003) Equilibrium study of selected divalent d-electron metals adsorption on A-type zeolite. J Colloid Interface Sci. 262: 321-330.

Masters DG, Keen CL, Lönnerdal B, Hurley LS (1983) Comparative aspects of dietary copper and zinc deficiencies in pregnant rats. J Nutr. 113: 1448-1451.

McArdle HJ (1995) The metabolism of copper during pregnancy—a review. Food Chemistry 54: 79-84.

Medeiros DM, Bagby D, Ovecka G, McCormick RJ (1991) Myofibrillar, mitochondrial and valvular morphological alterations in cardiac hypertrophy among copper deficient rats. J Nutr 121: 815–824.

Medeiros DM (2016) Copper, iron, and selenium dietary deficiencies negatively impact skeletal integrity: A review. Exp Biol Med (Maywood) 241: 1316-1322.

Medeiros DM (2017) Perspectives on the role and relevance of copper in cardiac disease. Biol Trace Elem Res. 176: 10-19. doi: 10.1007/s12011-016-0807-z.

Melvin P et al. (2021) Silver Acetate, Silver Nitrate, Micron-sized Silver and Nanoparticulate Silver: A Comparative Toxicokinetic Study in Rats by Single and Repeat Administration. Labcorp Study Number: CC71MP.

Nelson KT, Prohaska JR (2009) Copper deficiency in rodents alters dopamine beta-mono-oxygenase activity, mRNA and protein level. Br J Nutr. 102: 18-28.

Nose Y, Kim BE, Thiele, DJ (2006) Ctr1 drives intestinal copper absorption and is essential for growth, iron metabolism, and neonatal cardiac function. Cell. Metab. 4: 235–244.

Olivetti G, Quaini F, Lagrasta C, Ricci R, Tiberti G, Capasso JM, Anversa P (1992) Myocyte cellular hypertrophy and hyperplasia contribute to ventricular wall remodeling in anemia-induced cardiac hypertrophy in rats. Am J Pathol. 141: 227-239.

Oster G, Salgo MP (1977) Copper in mammalian reproduction. Adv. Pharmacol. Chemother. 14: 327-409.

Patchin ES, Anderson DS, Silva RM, Uyeminami DL, Scott GM, Guo T, Van Winkle LS, Pinkerton KE (2016) Sizedependent deposition, translocation, and microglial activation of inhaled silver nanoparticles in the rodent nose and brain. Environ Health Perspect. 124: 1870-1875.

Penland JG, Prohaska JR (2004) Abnormal motor function persists following recovery from perinatal copper deficiency in rats. J Nutr. 134: 1984–1988.

Prohaska JR, Heller LJ (1982) Mechanical properties of the copper-deficient rat heart. J Nutr. 112: 2142-2150.

Prohaska JR, Downing SW, Lukasewycz OA (1983) Chronic dietary copper deficiency alters biochemical and morphological properties of mouse lymphoid tissues. J Nutr. 113: 1583-1590.

Prohaska JR, Hoffman RG (1996) Auditory startle response is diminished in rats after recovery from perinatal copper deficiency. J Nutr 126: 618–627.

Prohaska JR (2000) Long-term functional consequences of malnutrition during brain development: copper. Nutrition. 16: 502-504.

Pyatskowit JW, Prohaska JR (2008) Copper deficient rats and mice both develop anemia but only rats have lower plasma and brain iron levels. Comp Biochem Physiol C Toxicol Pharmacol. 147: 316-323.

Ren F, Logeman BL, Zhang X, Liu Y, Thiele DJ, Yuan P (2019) X-ray structures of the high-affinity copper transporter Ctr1. Nature communications, 10 [1386]. https://doi.org/10.1038/s41467-019-09376-7.

Renaut R et al. (2022) Silver Acetate: Extended One Generation Reproductive Toxicity Study in the Sprague Dawley Rat by Dietary Administration. Labcorp Early Development Laboratories Ltd. Labcorp Study Number 8437234 (draft audited report).

Renaut R et al. (2021) Silver Acetate: Preliminary Reproductive Performance Study in the Sprague Dawley Rat by Dietary Administration. Labcorp Early Development Laboratories Ltd. Labcorp Study Number 8436495 (draft audited report).

Rungby J, Danscher G (1983a) Neuronal accumulation of silver in brains of progeny from argyric rats. Acta Neuropathol. 61: 258-262.

Rungby J, Danscher G (1983b) Localization of exogenous silver in brain and spinal cord of silver exposed rats. Acta Neuropathol. 60: 92–98.

Rungby J, Slomianka L, Danscher G, Andersen AH, West MJ (1987). A quantitative evaluation of the neurotoxic effect of silver on the volumes of the components of the developing rat hippocampus. Toxicology 43: 261–268.

Shavlovski MM, Chebotar NA, Konopistseva LA, Zakharova ET, Kachourin AM, Vassiliev VB, Gaitskhoki VS (1995) Embryotoxicity of silver ions is diminished by ceruloplasmin – further evidence for its role in the transport of copper. BioMetals 8: 122-128.

Skalska J, Frontczak-Baniewicz M, Strużyńska L (2015) Synaptic degeneration in rat brain after prolonged oral exposure to silver nanoparticles. Neurotoxicology 46:145-154.

Sprando RL, Black T, Keltner Z, Olejnik N, Ferguson M (2017) Silver acetate exposure: Effects on reproduction and post natal development. Food Chem Toxicol. 106: 547-557.

Tang J, Xiong L, Wang S, Wang J, Liu L, Li J, Yuan F, Xi T (2009) Distribution, translocation and accumulation of silver nanoparticles in rats. J Nanosci Nanotechnol. 9: 4924–4932.

Sugawara N, Sugawara C (2000) Competition between copper and silver in Fischer rats with a normal copper metabolism and in Long-Evans Cinnamon rats with an abnormal copper metabolism. Arch Toxicol. 74:190–195.

Uriu-Adams JY, Keen CL (2005) Copper, oxidative stress, and human health. Mol Aspects Med. 26: 268-298.

Uriu-Adams JY, Scherr RE, Lanoue L, Keen CL (2010) Influence of copper on early development: prenatal and postnatal considerations. Biofactors 36: 136-152.

US National Research Council [US NRC] (1995) Nutrient Requirements of Laboratory Animals,: Fourth Revised Edition, 1995. Washington, DC: The National Academies Press (US).

US National Research Council [US NRC] (2000) Committee on Copper in Drinking Water. Copper in Drinking Water. Washington (DC): The National Academies Press (US).

Williams DM (1983) Copper deficiency in humans. Semin Hematol. 20: 118-128.

Yu L, Liou IW, Biggins SW, Yeh M, Jalikis F, Chan LN, Burkhead J (2019) Copper deficiency in liver diseases: a case series and pathophysiological considerations. Hepatol Commun. 3: 1159-1165.

Zatta P, Frank A (2006) Copper deficiency and neurological disorders in man and animals. Brain Res Rev. 54: 19-33.

Zheng W (2001) Toxicology of choroid plexus: special reference to metal-induced neurotoxicities. Microsc Res Tech. 52: 89–103.

Zimmerman AW, Matthieu JM, Quarles RH, Brady RO, Hsu JM (1976) Hypomyelination in copper-deficient rats. Prenatal and postnatal copper replacement. Arch Neurol. 33: 111-119.

Zimnicka AM, Maryon EB, Kaplan JH (2007) Human copper transporter hCTR1 mediates basolateral uptake of copper into enterocytes: implications for copper homeostasis. J Biol Chem 282: 26471–26480.

Zucconi GG, Cipriani S, Scattoni R, Balgkouranidou I, Hawkins DP, Ragnarsdottir KV (2007) Copper deficiency elicits glial and neuronal response typical of neurodegenerative disorders. Neuropathol Appl Neurobiol. 33: 212-225.

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