Minority position on Silver : Mutagenicity and Fertility – Wendy Rodriguez

Read across approach

In the RAC opinion on Silver (bulk and nanoparticles), different weight is given to the same read-across approach depending on the analyzed endpoint, whereas such different approach is not scientifically justified. In particular, data with a similar level of quality, obtained with silver acetate (AgOAc), are considered supportive in STOT RE and mutagenicity endpoints but are disregarded for fertility and development (based on the assumption of a different Ag^+ bioavailability when comparing target-substance and AgOAc), which lead to **inconsistencies within the opinion.**

Based on current data and the scope of the CLH Entry (Ag massive, powder and nanoparticles), I am of the opinion that the variations in bioavailability between target-substances and AgOAc as a reason to dismiss study results for reproductive toxicity assessment is not scientifically justified. I support using a read-across approach based on AgOAc **as <u>supportive</u> evidence** for the reproductive toxicity assessment, for the following reasons :

- *The CLH Entry for Silver covers a wide range of particles sizes, which impacts the bioavailability*
  The CLH classification for Silver covers both microparticles (AgMP) and nanoparticules (AgNPs) without distinction. But AgMP are not expected to show the same bioavailability than AgNPs, neither all the AgNPs between them, as illustrated in Boudreau et al. (2016) and Anon. (2021), which means that a wide range of bioavailability was considered from the very beginning.

- *The assumption of a considerable difference between Ag^+ bioavailability after exposure to AgOAc vs. Silver (AgNPs and AgMP) is not scientifically justified.*
  The read across with AgOAc is not supported for reprotoxicity (fertility and development) in RAC opinion, based on a difference of Ag^+ release assumed to be too disproportionate when compared with Silver (AgNPs and MP). This assumption mainly rely on two toxicokinetic studies, Anon. (2021) and Boudreau and al. (2016).
  Boudreau et al. (2016, Fig 3B), shows that silver blood levels (12h) in males rats after oral exposure to AgOAc and to $AgNP_{10nm}$ are much more similar between them (almost equivalent) than when compared with silver blood levels (12h) after exposure to $AgNP_{75nm}$ and AgMP. In females, the difference between Ag blood levels (12h) after exposure to $AgNP_{75nm}$ and $AgNP_{10nm}$ is really similar to the ones between rats exposed to $AgNP_{10nm}$ and AgOAc (Fig 3A). In addition, the mean concentration of Ag in blood (6h) is in the same order of magnitude in rats after AgOAc and AgNP exposure (at low doses), as indicated in Anon. (2021). This is especially true in males, where the Ag (6h) concentration in blood is equivalent after exposure to both substances.

Mutagenicity

I consider that Silver should be classify for germ cell mutagenicity, **category 2** for the following reasons:

- *A gene mutation profile is clearly highlighted in vitro*
  The complete dataset shows a clear majority of positive mammalian gene mutations test. In addition, out of 10 *in vitro* Comet assays, none demonstrated negative results.
  The overall weight-of-evidence shows a profile of gene mutation in vitro.

- *A global assessment of all in vivo tests altogether is not in accordance with the CLP guidance - In vivo micronucleus assays do not detect gene mutations*
  The main negative in vivo evidences come from **micronucleus** (table 12 of the RAC opinion), which **do not detect gene mutation** and therefore should not be used to disregard positive in vitro results of gene mutation tests or comet assays.
  This view is supported by the CLP guidance (page 366; ECHA, 2017) stating that: "*A complex data situation with positive and negative results might still lead to classification. This is because all tests detecting a certain type of mutation (e.g. point mutations) have been positive and all tests detecting chromosome mutations have been negative.* ***Such circumstances clearly warrant classification although several tests have been negative*** *which is plausible in this case.*"

- *The overall in vivo Comet assay dataset corroborate the positive in vitro findings.*
  An in-depth evaluation of in vivo studies demonstrates a positive **weight-of-evidence for** the **Comet assay dataset.**
  - Four in vivo Comet assays, performed with different species exposed to AgNPs, showed **statistically significant positive results** (Patlolla et al., 2015; Al Gurabi et al., 2015; Awasthi et al., 2015 ; Gromadzka-Ostrowska et al., 2012), three of them being **dose-dependent** as well. In two studies, the animals were exposed orally, increasing the concern. Moreover, positive γ-H2AX immunofluorescence assay (Kovvuru et al., 2015) was also reported, and can be considered as supportive. Li et al. (2014) study includes two types of Comet assay (alkaline and enzyme modified) performed on mice exposed to AgNPs and shows equivocal results.
  - On the other hand, from the **3 remaining** *in vivo* Comet assays concluded negative, two of them showed **concerning limitations** that **impact the reliability** of the final results. The first one (IIIA 6.6.5-02) was performed with silver zinc zeolite, an ion exchanger resin which is not supported for Silver read-across. The second comet assay (Dobrzyńska et al., 2014) was lacking positive controls, therefore questioning the relevance of the negative results.

- *The in vivo Comet assay is adequate to support a classification on germ cell mutagenicity Cat.2*
  The CLP (Regulation (EC) No 1272/2008; Annex I: 3.5.2.2; Table 3.5.1) allocates the classification of a mutagen in category 2 based on "*Positive evidence obtained from experiments in mammals and/or in some cases from in vitro experiments, obtained from: Somatic cell mutagenicity tests in vivo, in mammals;* ***or other in vivo somatic cell genotoxicity tests*** *which are supported by positive results from in vitro mutagenicity assays*." The CLP

guidance (page 363; ECHA, 2017) states that: "***In vivo tests in somatic cells*** *which provide information on* ***genotoxicity include****, for example, the* ***Comet single cell gel electrophoresis assay*** *for DNA strand breaks."*

- *There are indications that Silver may induce heritable mutations in human germ cells*
  Although no reliable germ cell mutagenicity assay was made available for the assessment of Silver, data from the CLH report raise that silver may induce heritable mutations in the germ cell of humans. An in vivo Comet assay demonstrated positive results in rat full testes after exposure to AgNPs (Gromadzka-Ostrowska et al., 2012). In addition, the majority of the data from the CLH dossier indicates an adverse effect on males germ cells in several species (see "fertility" part of this position). Finally, AgNPs clearance appears low in testes (Lee and al., 2013; Van der Zande et al., 2012) and a dose-dependent deposition in testes was detected in rat after oral and inhalation exposure to AgNPs (Kim and al., 2008; Kim and al., 2011)

To conclude, there is evidences that Silver can cause gene mutations *in vitro*. Although, *in vivo* micronucleus are mainly negatives, this type of test is not adequate to detect gene mutations. **The weight-of-evidence** points toward **positive results in Comet** assays *in vivo*. In addition, there are several indications that Silver could reach and induce damages in male germ cells *in vivo*. Therefore, a classification as **mutagen category 2** is warranted in my opinion.

Reproductive toxicity: Sexual function and fertility

I support a classification **category 1B** for fertility, for the following reasons:

- ***Effects on sperm are consistently reported*** *in studies relevant for toxicity on sexual function and fertility*
  - Eight different studies stressed adverse effect on sperm after **AgNPs exposure**. A decrease of sperm cells at different levels of maturity (Miresmaeili et al., 2013), sperm cell morphology and motility alterations (Mathias et al., 2015; Baki et al., 2014; Amraie et al., 2013; Castellini et al., 2014) as well as a decrease of acrosome reaction/ increase damages (Mathias et al., 2015) and decrease of Leydig cells number (Baki et al., 2014) were described. These findings were **all statistically significant and dose dependant** (except in Castellini et al., 2014, as only one dose was tested). A statistically significant increase of abnormal epididymal spermatozoa and a significant decrease of sperm count were also find in Gromadzka-Ostrowska et al. (2012) study whereas Thakur et al. (2014) study described depletion of germ cells and germinal cells necrosis. In one **OECD TG 408 study**, statistically significant higher sperm total abnormalities were also reported at mid dose after AgNPs exposure (Lafuente et al., 2016).
  - Where sperm parameters were investigated, **none of the AgNPs studies reported was negative**
  - These effects were seen in different species (mice, rats, rabbits), in absence of general toxicity. In addition, persistence on testis and a low clearance were observed in two

studies (Lee and al., 2013; Van der Zande et al., 2012) as well as a dose dependant deposition of silver in testes after both oral and inhalation exposure in rats (Kim et al., 2007 and Kim and al. 2011). All this findings increase the concern.

- One OECD EOGRTS performed with AgOAc (Anon., 2022) according to GLP and OECD TG 443 guidance was available. Low mean absolute testes weight was described in mid and high dose group, as well as a treatment related effect on spermatid total millions, cauda epididymis sperm count and total millions (high dose group). In F1 pups exposed to an high dose of AgOAc, an effect on cauda epididymal spermatid counts as well as an increased incidence of abnormal morphology were also reported. This study should be considered as supportive.

- *The results published in peer reviewed scientific literature should be seen as relevant for classification in this case*
  The results consistently points toward an adverse effect on sperm, with high persistence of Silver in testis. There is no clear evidence that would allow to reject each of the studies from published literature. In addition these studies are supported by read across study that was performed with AgOAc according in respect to OECD guidance. I disagree with the assumption that the release of Ag+ would be too disproportionate after AgOAc exposure to allow to consider studies performed with that salt as *supportive* for reproductive effects seen in the context of AgMP and AgNPs classification (see read across part of this position).

- *Alteration of sperm parameters should be considered as an adverse effect on its own, independently of other fertility parameters*
  An adverse effects on sexual function and fertility is defined by the Annex I: 3.7.1.3 of CLP (Regulation (EC) No 1272/2008) in the following way: "*This includes, but is not limited to, alterations to the female and male reproductive system, adverse effects on onset of puberty,* ***gamete production and transport****, reproductive cycle normality, sexual behaviour, fertility, parturition, pregnancy outcomes, premature reproductive senescence, or modifications in other functions that are dependent on the integrity of the reproductive systems*."
  The apparent absence of effect on fertility index in rats does not decrease the concern for human as rats have a higher sperm reserve than humans. In addition, the World Health Organization classifies patients according to descriptive analyses of sperm number, motility and morphology as being the most common form of male infertility.
  No data has been provided indicating that relevance to human can be excluded, and decrease of sperm count and mobility alterations are relevant effects to classify.

- *Adverse effects on female reproduction were also reported*
  Although less investigated in the available studies, alteration of females gametes were also described in rat (follicle number decrease, increase of atretic and degenerated follicles, see Ema et al., 2017) after AgNPs exposure. Decreases in implantation and in females fertility index were also observed after AgOAc exposure (Sprando et al., 2016). Taking together, adverse effects on females reproduction can be considered as supportive for classification.

To conclude, when sperm parameters where analyzed, related adverse effects were identified among all the studies reported in the fertility part of the RAC opinion. And this, although these studies used

different animal species, strains, types/durations of exposure and different AgNPs size, vehicles, and preparations. This consistency of result increase the concern, and make the relevance to human very plausible. This overall dataset is robust, due to the consistency of adverse effect in literature (including one OECD TG 408 study), the lack of scientific reasons to dismiss each of them, and is supported by adverse effect reported in Anon. (2022) after exposure to AgOAc. Adverse effects on sperm are relevant for classification, according to the CLP guidance. Therefore, in my opinion, the overall weight-of-evidence warrant a **classification 1B**.

NB for developmental toxicity endpoint: The final RAC opinion concludes that "RAC considers there is insufficient data for an assessment of silver developmental toxicity and therefore does not propose classification for development for silver." The exact agreement, as reported in the Minutes of RAC-61 plenary for the development part is : "**RAC agreed on no classification based on inconclusive data**."