

Brussels, April 23, 2018

Study design: effects of soluble silver on the gut bacterial microbiota (v2)

Animals - Four week-old male (40) and female (40) Wistar rats will be purchased from Janvier Labs (St Berthevier, France), and kept at the local animal facility (Animalerie Centrale, Université catholique de Louvain, Brussels, Belgium) under SPF-like conditions in a controlled environment (22°C, 55% relative humidity, 16-h light/8-h dark cycle, with acidified water and food ad libitum) in autoclaved air-filtered polycarbonate cages with conventional sawdust (Carfil, Oud-Turnhout, Belgium).

Upon receipt, all animals will be identified by individual ear tags.

During two weeks before exposure, litters will be mixed every day to homogenize the baseline gut microbiota across the cages [1].

Rats will then be randomly assigned to experimental groups (2 rats/cage, 5 cages/dose group) and fed with control or AgAc-supplemented pellets during 10 wk.

Target treatment doses are 0, 0.4, 4 and 40 mg AgAc/kg bw/d based on [2].

Food - AgAc will be incorporated in food pellets by Carfil (Maintenance diet, Turnhout, Belgium). Considering an average weight of 200 or 300 g, and based on a daily average consumption of 20 or 30 g food/d for M or F, respectively, concentrations of AgAc in pellets will be 4, 40 and 400 mg AgAc/kg. Preliminary studies will be conducted to verify the stability of AgAc in the food. This will be assessed at LTAP by measuring the solubility of Ag from pellet powder supplemented with the lowest dose (4 mg AgAc/kg) over a period of 28 d [3].

Exposure – If no stability issue is documented over a period of 28 d, fresh batches of AgAc pellets will be prepared by Carfill at the start of exposure, after 4 and 8 wk of exposure (each batch will be used during max. 28 d). If AgAc does not appear stable in food, another exposure scenario will need to be discussed.

Animals will be weighted at the beginning of the exposure and 1 x/week along the exposure and just before sacrifice.

Food consumption will also be recorded 3 x/week per cage to calculate actual AgAc daily intake.

After 28 d, blood will be collected from the tail vein (EDTA tubes) and a fraction will be immediately centrifuged. Whole blood and plasma will be stored at -20°C for chemical analysis. 24 h-feces will be collected in cages with new bedding; the rats will be fasted during this period to avoid contamination of the feces by the food. Samples will be stored at -20°C for Ag analysis.

After 10 wk exposure, rats will be euthanized by an intra-muscular injection of pentobarbital and intra-cardiac blood will be collected on EDTA, centrifuged and stored as above. Left ovary and uterus, left testis, brain, thymus, liver, kidney, spleen will be collected, weighted and stored in PFA for possible histopathological examination (to be decided later). Right ovary and uterus, right testis will be weighted and stored frozen for Ag determination.

At the end of the necropsy,

Ileum and colon tissue will be collected and immediately stored in PFA (optional histopath) and in Tripure for subsequent transcriptomic analysis (optional RNA Seq).

Fecal samples will be collected per rat and stored at -20°C before extraction with a QIAamp DNA Stool Mini kit (Qiagen, Hilden, Germany) and stored at -20°C until next generation sequencing (NGS) analysis at MR DNA lab (Shallowater, USA). The gut microbial populations will be identified and quantified on an Illumina MiSeq platform with specific primers of the bacterial 16S rRNA gene (2x300bp PE Illumina 20,000 sequence diversity assay). Raw sequences will be processed using USEARCH v8.0.1623 (Tiburon, USA) to generate operational taxonomic units (OTU). The taxonomic assignment of each OTU will be carried out using a local BLAST against the Greengenes database version 13.5. Analyses of the α - (richness and evenness within a microbial community) and β - (differences in composition among communities) diversities and statistical analyses will then be performed using the R software (version 3.1.2., Vienna, Austria) as described in [3].

Ag content will be measured by inductively coupled plasma mass spectrometry (ICP-MS) in whole blood, feces (28 d), and whole blood, ovary, uterus/testis and intestinal feces (10 wk). The methodology described in van der Zande (2012) will be followed for tissue preparation, mineralization and analysis. Fecal materials will probably also have to be mineralized before analysis.

Cu and Se will be measured by ICP-MS in plasma at 28 d and wk 10.

Plasma ceruloplasmin (ferroxidase) activity will be measured in plasma (28 d and 10 wk) by a colorimetric kit (Sigma, St Louis, USA).

References

1. Ma BW, Bokulich NA, Castillo PA, Kananurak A, Underwood MA, Mills DA *et al.*: Routine habitat change: a source of unrecognized transient alteration of intestinal microbiota in laboratory mice. *PLoS One* 2012, 7: e47416.
2. Sprando RL, Black T, Keltner Z, Olejnik N, Ferguson M: Silver acetate exposure: Effects on reproduction and post natal development. *Food Chem Toxicol* 2017, 106: 547-557.
3. van den Brule S, Ambroise J, Lecloux H, Levard C, Soulas R, De Temmerman PJ *et al.*: Dietary silver nanoparticles can disturb the gut microbiota in mice. *Part Fibre Toxicol* 2016, 13: 38.
4. van der Zande, M. *et al.* Distribution, elimination, and toxicity of silver nanoparticles and silver ions in rats after 28-day oral exposure. *ACS Nano* 2012,6:7427-7442