Universidad de Navarra

Facultad de Farmacia y Nutrición

Departamento de Farmacología y Toxicología

TESIS DOCTORAL

Biosafety evaluation of an antiseptic formulation containing silver nanoparticles

Evaluación de la bioseguridad de una formulación antiséptica que contiene nanopartículas de plata

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Memoria presentada por D. ª Adriana Rodriguez Garraus para aspirar al grado de Doctor por la Universidad de Navarra.

Fdo. Adriana Rodriguez Garraus

Pamplona, a 14 de marzo de 2022

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El presente trabajo de investigación titulado:

Biosafety evaluation of an antiseptic formulation containing silver nanoparticles

que presenta Dña. Adriana Rodriguez Garraus para aspirar al grado de Doctor por la Universidad de Navarra, ha sido realizado en el Departamento de Farmacología y Toxicología de la Facultad de Farmacia y Nutrición, bajo la dirección de las Dras. Adela López de Cerain Salsamendi y Amaya Azqueta Oscoz. Considerando finalizado el trabajo, autorizan su presentación a fin de que pueda ser juzgado y calificado por el Tribunal correspondiente.

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List of abbreviations

AgNPs	Silver nanoparticles
AMR	Antimicrobial resistance
ALP	Alkaline phosphatase
ALS	Alkali-labile sites
ALT	Alanine amino transferase
AOT	Sodium bis(2-ethylhexyl)-sulfosuccinate
AST	Aspartate amino transferase
BEUC	Bureau Européen des Unions de Consommateurs
BSA	Bovine serum albumin
CE	Cloning efficiency
CHO	Cholesterol
CNRS	Centre National de la Recherche Scientifique
CPK	Creatine phosphokinase
CREA	Creatinine
DAPI	4,6-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
EFSA	European Food Safety Authority
EMA	Ethidium monoazide bromide
NCE	Normochromic erythrocytes
Endo-III	Endonuclease-III
PCE	Polychromatic erythrocytes
ERDF	European Regional Development Fund
FBS	Fetal bovine serum
Fpg	Formamide pyrimidine DNA-glycosylase
GEF	Global evaluation factor
GLPs	Good laboratory practices
GLU	Glucose
HIHS	Heat inactivated horse serum
HPRT	Hypoxanthine-guanine phosphoribosyl transferase
ICH	International Council for Harmonization

ISO	International Organization for Standardization
KO	Knockout
LMP	Low melting point
MF	Mutant frequency
MIRCA	Minimum information for reporting comet assay
MLA	Mouse lymphoma assay
MMS	Methyl methane sulfonate
MN	Micronucleus
MPS	Mononuclear phagocyte system
MRD	Maximum repeated dose
NMs	Nanomaterials
NPs	Nanoparticles
OECD	Organisation for Economic Co-operation and Development
OGG-1	Oxoguanine glycosylase-1
PBS	Phosphate buffered saline
PTT-a	Prothrombin time
PVP	Polyvinylpyrrolidone
RCE	Relative cloning efficiency
ROS	Radical oxygen species
RSG	Relative suspension growth
RTG	Relative total growth
SBs	Strand breaks
SD	Standard deviation
SSC	Side scatter
ST	Standard
TFT	5-trifluorothymidine
TG	Triglycerides
TK	Thymidine kinase
TSG	Total suspension growth
WHO	World Health Organization
XPRTT	Transcriptional activator of protease

Chapter 1. General introduction

1. Antimicrobial resistance

According to the World Health Organization (WHO) and the United Nations, antimicrobial resistance (AMR) has been stated as one of the biggest threats to global public health, posing serious security and economic problems (Bulteel et al., 2021; Dadgostar, 2019; Shankar, 2016; United Nations, 2016; World Health Organization, 2015). Some of the health improvements achieved in recent years, as infectious diseases reduction, diseases community prevention, access to health services, safe, efficacious and quality medicines, safe water, and healthy food, are being challenged by AMR (United Nations, 2016). Resistant pathogens began to originate worldwide in the 1980s and have emerged against almost every antibiotic existing, due to their indiscriminate and extensive usage during the last 70 years (Davies and Davies, 2010; Samreen et al., 2021; Xiong et al., 2018).

One of the main reasons of AMR development is the therapeutic and non-therapeutic overuse of antimicrobials in humans and non-humans (Palma et al., 2020; Shankar, 2016; World Health Organization, 2015). The non-human use of antimicrobials includes food and companion animals, aquaculture and horticulture, and can be destined to therapeutic, prophylactic, metaphylactic and growth promotion purposes (WHO and FAO, 2003). A large number of the antibiotics used for the treatment of various human infectious diseases are also commonly used in the aquaculture and livestock industries (Samreen et al., 2021). Initially, veterinary medicines were destined to treat animal infections but now they are also used as prophylactic agents, feed additives and growth promoters (Samreen et al., 2021). The prophylactic use in animal production as growth promoters consists in the long-time administration of subtherapeutic antimicrobial doses, and is an important contributor to antimicrobial resistance occurrence and its spread between animals, humans, and environment (Cheng et al., 2019; Palma et al., 2020; Vidovic and Vidovic, 2020; WHO and FAO, 2003).

The Global Leaders Group on AMR have called upon all countries to reduce the levels of antimicrobial use in food systems, including the ones used in animal growth promotion ("World leaders and experts call for significant reduction in the use of antimicrobial drugs in global food systems"). Moreover, the WHO published an online document in which world leaders and experts call for significant reduction in the use of antimicrobial drugs in global food systems ("World leaders and experts call for significant reduction in the use of antimicrobial drugs in global food systems"). Despite measures taken by some WHO Member States, antibiotic use in humans, animals and agriculture is still increasing globally. Thus, every nation is potentially at risk due to the misuse of antimicrobials for animal or human health, and also in the agriculture, animal and aquaculture production (United Nations, 2016; World Health Organization, 2015).

The appearance of AMR caused by the use of antibiotics in animal feed was detected years ago and solutions have been explored since then. In order to assure the prudent usage of drugs destined to treat human infections, environmental policies are making efforts to minimize or even restrict their application in growth promotion

(Samreen et al., 2021). The United Kingdom Joint Committee on the Use of Antibiotics in Animal Husbandry released the Swann Report in 1969, recommending for growth promotion only the antibiotics of little or no application as therapeutic agents ("House of Lords - Science and Technology - Seventh Report,", Samreen et al., 2021). Furthermore in 1999, the European Commission highlighted the need to face out the antimicrobials use as growth promoters and finally, their banishment entered into force in 2006 (Arsène et al., 2021; European Parliament and the Council of the European Union, 2003; UE, 2006). A few years later, in 2017, the same ban was also established in the USA (Samreen et al., 2021; World Organisation for animal health, 2021). The impossibility of using antibiotics in animal feed raises an urgent need for alternatives. One of the strategies in Europe to face this lack of alternatives is to finance projects whose purpose is to replace the use of antibiotics in animal feed by new materials.

2. OUTBIOTICS project

One of the projects focused on the reduction of the AMR problem by contributing to the replacement of antibiotics is the POCTEFA 2014-2020 "Innovative technologies for diagnosis, prevention and elimination of emerging pollutants (antibiotics) from the waters of the POCTEFA territory (EFA 183-16)" so called OUTBIOTICS ("Proyecto Outbiotics – (POCTEFA 2014 – 2020)"). POCTEFA 2014-2020 is the acronym of the INTERREG V-A Spain-France-Andorra Program, a cooperative European programme of territorial collaboration to promote the sustainable development of the border territory between the three countries (POCTEFA region). POCTEFA 2014-2020 is the 5th generation of financial support to the communities, destined to reinforce the economic and social integration within the zone. OUTBIOTICS is also co-financed by the "European Regional Development Fund" (ERDF).

The POCTEFA zone is composed by regions of Navarra, Huesca, Zaragoza, Lérida, Pyrénées Atlantiques, Hautes-Pyrénées, Pyrénées-Orientales, Haute-Garonne, and Ariège. Antibiotics pose an especially pressing impact to the environment in the areas of livestock farms in the POCTEFA region. Intensive livestock farming is one of the main economic engines in the rural regions of POCTEFA territory, and it is one of the biggest responsible of antibiotic emissions in the POCTEFA region.

OUTBIOTICS project integrates partners from both sides of the Pyrenees being:

1. University of Zaragoza: "Group of Analytical Spectroscopy and Sensors", "Group of Quality and Water Treatment", "Joint University Agri-food Research Institute" and "Group of Animal Production".
2. University of Navarra: "Pharmacology and Toxicology Department" and "Microbiology and Parasitology Department".

3. University of Lleida: “Animal Science Department” and “Higher Technical School of Agricultural Engineering”, “Department of chemistry - Physicochemistry of Macromolecules of Environmental Interest”.
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The project comprises three specific objectives: 1) the diagnosis and quantification of the amount of antibiotics in POCTEFA waters near livestock farms from 2018 to 2020, 2) the replacement of antibiotics in livestock production with bactericidal nanomaterials (NMs), and 3) the development of new water treatment tools to eliminate antibiotics. Thus, one important objective of OUTBIOTICS project is to eliminate or considerably reduce antibiotics in the POCTEFA territory waters, through the development of innovative technologies based on inert nano and micro-materials. These materials will be used to replace antimicrobials in animal feed and will prevent their emission into the environment.

Within the specific objectives of the project, this PhD thesis is framed in the development of new bactericides based on NMs to replace or reduce the use of antibiotics in livestock farms of the POCTEFA territory. A new material consisting of a metallic silver-based clay has been developed, grounded in the known bactericidal activity of silver, to be used as feed additive.

Within the specific objectives of the project, this PhD thesis is framed in the development of new bactericides based on NMs to replace or reduce the use of antibiotics in livestock farms of the POCTEFA territory. A new material consisting of a metallic silver-based clay has been developed, grounded in the known bactericidal activity of silver, to be used as feed additive. A method under patent (ENOSAN) through which commercial kaolin is treated, enables silver (Ag) to be deposited on its surface as silver nanoparticles (AgNPs). The resulting material is a kaolin formulation containing AgNPs embedded in its surface (Feed additive for animals: Spanish National patent Nº 200701496 and Nanosystems comprising silver and antibiotics and their use for the treatment of bacterial infections: Patent in international extension China, Japan, USA, EPO, Brazil, Colombia and Mexico, Nº PCT/EP2018/059006). The use of the silver-kaolin formulation in livestock sector as an alternative to antibiotics in farms, would contribute to decrease antibiotics overuse in animals and with it, the AMR problem associated. An important part of an additive development is the safety assessment, and this is the objective in which the PhD thesis has been framed.

3. Nanotechnology

Nanotechnology has become one of the most sought after technologies in a range of scientific fields and has been identified as a key enabling technology that provides the basis for further innovation and new products (The European Commission, 2012a). However, there is currently no standard definition of NMs. Each specific piece of legislation covers only the NMs within its scope; therefore, the definitions of NMs and a NPs can be used only in a defined regulatory context (Rauscher et al., 2017). According to the International Organization for Standarization (ISO), a NM is a "material with any external dimension in the nanoscale (nano-object) or having internal structure or surface structure in the nanoscale (nanostructured material)" and a nanoparticle is a "nano-object with all external dimensions at the nanoscale, where the length of the longest and the shortest axes of the nano-object do not differ significantly". Nanoscale is defined as a "length range approximately from 1 nm to 100 nm" (ISO/TS 80004-1:2015) (International Organization of Standarization, 2017). According to the European Commission, a NM is "a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm–100 nm" (The European Commission, 2011). Although there is no scientific justification for the 1–100 nm threshold, this is based on the fact that many of the particles within the size range, present the specific behaviour of NMs (The European Commission, 2012b).

The market offers a wide range of NPs, which vary according to their composition, size, shape, surface materials and other physicochemical characteristics. These materials are normally classified based on morphology, size or chemical characteristics.

4. Silver and silver nanoparticles as antimicrobial

Silver has been used for many purposes for thousands of years. Fields of application include jewellery, dental alloys, photography, explosives and because of its antimicrobial properties, drinking water disinfection (Chen and Schluesener, 2008; Nowack et al., 2011). Silver antimicrobial activity has been known since olden times. Greeks and Romans used silver containers to maintain water and other beverages in good conditions for long periods of time, and Australian and American pioneers inserted silver coins in their water and milk barrels (Etemadzade et al., 2016). Indeed, before the introduction of antibiotics, silver had been used to treat open wounds, burns, ulcers and eye infections since ancient times (Chen and Schluesener, 2008; Nowack et al., 2011; Silver et al., 2006). Its biocidal function was assumed to be associated with the release of Ag^+ ions.

In 1889, M.C. Lea synthesized a colloid of silver stabilized with citrate, with an average diameter of 7 to 9 nm (Carey, 1889). Those silver colloidal products with an average size of 10 nm were commercialized and applied

in medical field since 1897, named as Collargol (Fortescue-Brickdale et al., 1903). According to the product patent, Collargol must be a dispersion of particles with a colloidal size of less than 25 nm (Manes, 1968; Nowack et al., 2011), which falls within the nanoparticle range (1–100 nm). According to the ISO and the European Commission NPs definitions previously mentioned, Collargol could be considered as the first AgNPs. Collargol was broadly used in medicine during the first years of the 20th century but after the discovery of antibiotics its use was noticeably reduced (Medici et al., 2019). However, nowadays nanotechnology has emerged and AgNPs are widely used in several sectors (Table 1).

The use of AgNPs as antimicrobials is rising again due to the rapid growth of AMR (Medici et al., 2019; Schneider, 2017; Wong and Liu, 2010). Table 1 shows some of the wide applications of the AgNPs nowadays, being the antimicrobial activity one of the most important.

Table 1. Current applications of AgNPs

Property	Applications
Antimicrobial	Food sector Animal husbandry Filters for water Gynecological suppositories Wound dressings Catheters Contraceptic devices Orthopedic materials Drug-delivery systems Shower products Soaps Shampoos Anti-perspirants Facial cleansers and care products
Optical, Electrical, Thermal	Electronics Imaging Catalysis Biosensing
Morphology	Raman spectroscopy Fluorescence Analytics Sensing
Antiviral	Influenza

Information obtained from: Ardestani et al., 2015; Awad et al., 2021; Calderón-Jiménez et al., 2017; Dakal et al., 2016; El Mahdy et al., 2015; Evans et al., 2017; Franci et al., 2015; Geethalakshmi and Sarada, 2012; Giesz et al., 2017; Hebeish et al., 2014; Huma et al., 2018; Ibrahim, 2015; Kaur et al., 2019; Laux et al., 2017; Lee et al., 2018; W. Li et al., 2017; Mc Gillicuddy et al., 2017; Mikhailov and Mikhailova, 2019; Rai et al., 2012; Roszak et al., 2017; Sau et al., 2010; Schneider, 2017; Shaalan et al., 2017; Subashini et al., 2014; Tolaymat et al., 2010; Vila et al., 2018; Villeret et al., 2018; Wei et al., 2015; Wen et al., 2017; Wijnhoven et al., 2009; Xu et al., 2012; Yang et al., 2017.

The antimicrobial activity of AgNPs has been demonstrated, both alone and combined with antibiotics, against multidrug resistant bacteria (Hofer, 2019; Huma et al., 2018; Kaur et al., 2019). Several studies have confirmed the excellent antibacterial activity of AgNPs against resistant *Yersinia enterocolitica, Proteus vulgaris,*

Escherichia coli, Staphylococcus aureus, Enterococcus. faecalis, Staphylococcus epidermidis, Streptococcus pyogenes, Bacillus subtilis, Serratia marcescens, Citrobacter ssp., Klebsiella pneumoniae and *Pseudomonas aeruginosa*, being more pronounced against gram-negative bacteria than gram-positive ones (Dakal et al., 2016; Evans et al., 2017; Geethalakshmi and Sarada, 2012; Ibrahim, 2015; Lee et al., 2018; Namasivayam et al., 2011; Nanda and Saravanan, 2009; Subashini et al., 2014). Moreover, AgNPs have shown to enhance the activity of vancomycin against both *Staphylococcus aureus* and *Escherichia coli*, and of ampicillin, kanamycin, erythromycin, and chloramphenicol against gram-positive and gram-negative bacteria, being the enhancement of ampicillin the most pronounced one (Fayaz et al., 2010; Kaur et al., 2019).

Moreover, in the last years the development of materials based on clays with AgNPs attached on their surface has emerged, and it has been demonstrated that they maintain and even potentiate the AgNPs antimicrobial activity (Table 2). The immobilization of AgNPs on solid matrixes as kaolin or other clays to obtain silver-clay based materials, enhances the AgNPs stability, avoids their agglomeration, strengthens their antimicrobial activity by providing a large number of active surface sites and improves their biocompatibility (Hariram et al., 2021; Su et al., 2012, 2011; Unuabonah et al., 2018).

Table 2. AgNPs-clay based materials and their *in vitro* antibacterial effect; the effectiveness column compiles the bacteria against which the material has demonstrated antibacterial effect.

Material	Effectiveness	Reference
Silver-kaolin nanostructures	*Staphylococcus aureus, Bacillus subtilis, Escherichia coli*	(Hariram et al., 2021)
Silver-kaolin nanocomposites	*Enterococcus faecalis, Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Salmonella abony Pseudomonas aeruginosa*	(Awad et al., 2021)
AgNPs-kaolin	*Escherichia coli, Salmonella ssp.*	(Hassouna et al., 2017)
AgNPs-sodic montmorillonite	*Escherichia coli, Staphylococcus aureus*	(Abdel-Aziz et al., 2015)
Silver-silica core-shell nanoparticles	*Escherichia coli, Staphylococcus aureus*	(Devi et al., 2014)

Besides, although to a lesser extent, the antimicrobial efficacy of these materials has also been demonstrated in *in vivo* studies, suggesting that these materials could be used as a dietary supplement in animal feed. Thus, AgNPs supported in a sepiolite matrix given to pigs at 40 mg AgNPs/kg feed, produced a linear daily growth increase of the animals, together with a reduction in the population of ileal *Escherichia coli*, *Enterococcus faecalis* and *Salmonella enteritidis* (Fondevila, 2010; Latorre and Fondevila, 2010). Two years later, Su and colleagues (2012), carried out a study that confirmed that clays exert a slight antimicrobial activity and that they can potentiate AgNPs activity. Salmonella-infected chicks were fed with 10 mg/kg b.w. nano-silica platelets or

10 mg/kg b.w. AgNPs-nano-silica platelets increasing their survival rate to up to 50 % and even 75 %, respectively, in comparison with those who didn't receive any treatment, showing a survival rate of 10 % or less. Another study carried out in 2021, supports the use of silver-clay materials at low doses in animal feed; nano-silica-silver fed to broiler chickens at a dose 4 mg/kg b.w. for 35 days showed anti-inflammatory, antimicrobial, and immuno-stimulatory effects (Dosoky et al., 2021).

Also, the *in vitro* antibacterial activity of the silver-kaolin formulation developed in OUTBIOTICS was confirmed by Perez-Etayo and colleagues (Pérez-Etayo et al., 2021). The formulation exerted antimicrobial activity against a wide selection of gram-positive and gram-negative bacteria, including resistant and multi-resistant ones to different antibiotic groups, being more effective against gram-negative bacteria. Furthermore, it was found that the antibacterial effect *in vitro* was due to silver and not to kaolin. Kaolin formulation without silver was evaluated in the same conditions as silver-kaolin formulation, being ineffective against the microorganisms tested.

5. Safety evaluation of nanomaterials in animal feed

According to the European Food Safety Authority (EFSA), a feed additive is a product used in animal nutrition to exert an effect on animal feed, the animals, their derivative consumer products, or even the environment ("Feed additives, EFSA,"). Feed additives are usually applied to meet the need for a nutrient, increase the animal growth or improve the taste of feed ("Feed additives, EFSA,"). NMs can be included in the feed chain as feed additives (EFSA Scientific Comittee et al., 2018). However, when novel materials are introduced into food sector, it is essential to understand any impact they can cause on animal or human health. Therefore, a safety evaluation is required.

Safety evaluation of NMs is challenged due to their small size, large surface area, and unknown behaviour in biological systems (Butler et al., 2015). The EFSA published a guidance on human and animal health aspects of the risk assessment of nanoscience and nanotechnology applications in the food and feed chain in 2018 (EFSA Scientific Comittee et al., 2018). This guideline covers the evaluation of novel foods, food contact materials, food/feed additives and pesticides (EFSA Scientific Comittee et al., 2018). The EFSA guideline proposes a very complete risk assessment strategy for NMs evaluation, being one of the main sections the hazard identification (EFSA Scientific Comittee et al., 2018).

The hazard identification strategy suggested by the EFSA guideline consists of three steps (Figure 1) (EFSA Scientific Comittee et al., 2018). In the first place, it must be considered if the NMs are completely degraded in the gastrointestinal tract. If they are degraded, they must be evaluated through guidelines for conventional materials. The second step of the hazard identification corresponds to a revision of the available physicochemical and toxicological information. Also, an *in vitro* evaluation is suggested, consisting of lysosomal

dilution, genotoxicity and cytotoxicity studies. Finally, the third step entails the *in vivo* evaluation. The *in vivo* assessment includes a dose-finding study, followed by genotoxicity studies and a 90-day oral toxicity study. According to the EFSA guideline, every study must be carried out following it's corresponding Organisation for Economic Co-operation and Development (OECD) guideline. Once the evaluation is finished, if indications of slow elimination or specific distribution and accumulation in any tissue are found, further testing tailored to those results is recommended.

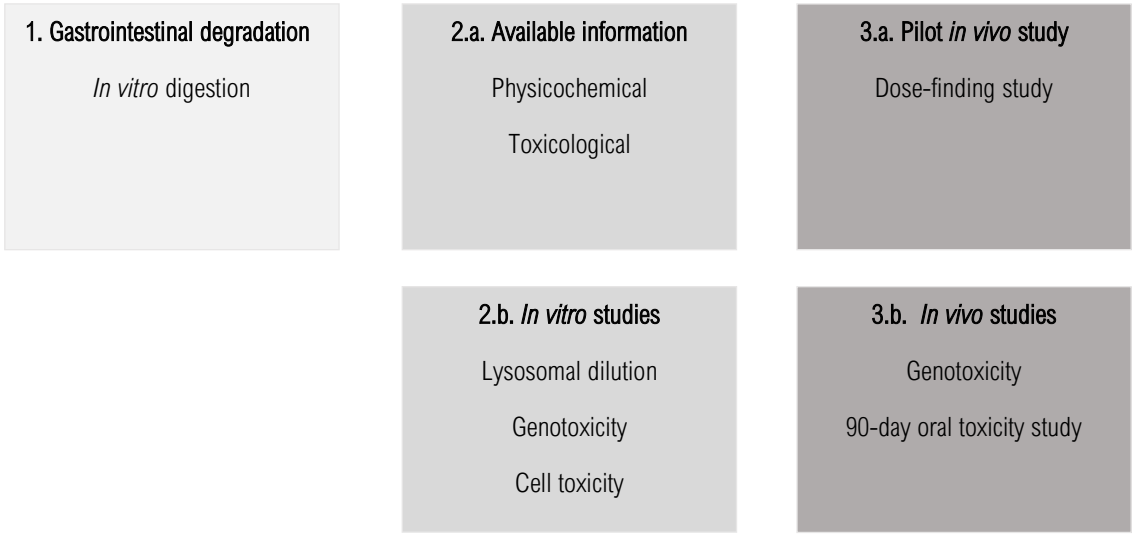

Figure 1. Scheme of the EFSA 2018 strategy for the hazard identification of NMs.

As can be seen in the Figure 2, genotoxicity of NMs must be evaluated in a sequential strategy that implies a first *in vitro* phase followed by a second *in vivo* phase, if necessary. In fact, it is essential within the risk and safety assessment of a compound, to identify potential mutagens and/or human carcinogens through the detection of primary DNA lesions, gene mutations and chromosomal damage. Genotoxicity tests enable the identification of risk regarding induction of DNA damage and fixation, and play an important role in the prediction of potential genotoxic and carcinogenic effects (Saks et al., 2017). DNA damage that is not repaired, or is mis-repaired, can lead to carcinogenicity or other diseases via the induction of mutations (Barnes et al., 2018; Magdolenova et al., 2014).

The *in vitro* genotoxicity strategy suggested by EFSA, covers the detection of the induction of gene mutations and chromosomal aberrations (Figure 2). For the study of the induction of gene mutations, two *in vitro* mammalian cell gene mutation tests are recommended: a) the hypoxanthine-guanine phosphoribosyltransferase (HPRT) or xanthine phosphoribosyl transferase (XPRT) gene mutation assay, following the OECD TG 476 (OECD, 1997a), and b) the mouse lymphoma thymidine kinase (TK) assay (MLA) following the OECD TG 490 (OECD, 2015). For the detection of structural and numerical chromosomal aberrations, it is suggested to carry out the *in vitro* mammalian cell micronucleus (MN) test, following the OECD TG 487 (OECD, 2014a). The *in*

vitro comet assay is not included in the strategy, but its modified version is mentioned as a complementary evaluation for the detection of oxidative DNA lesions (EFSA Scientific Comittee et al., 2018). This assay may provide supplementary information when testing materials that produce oxidative stress.

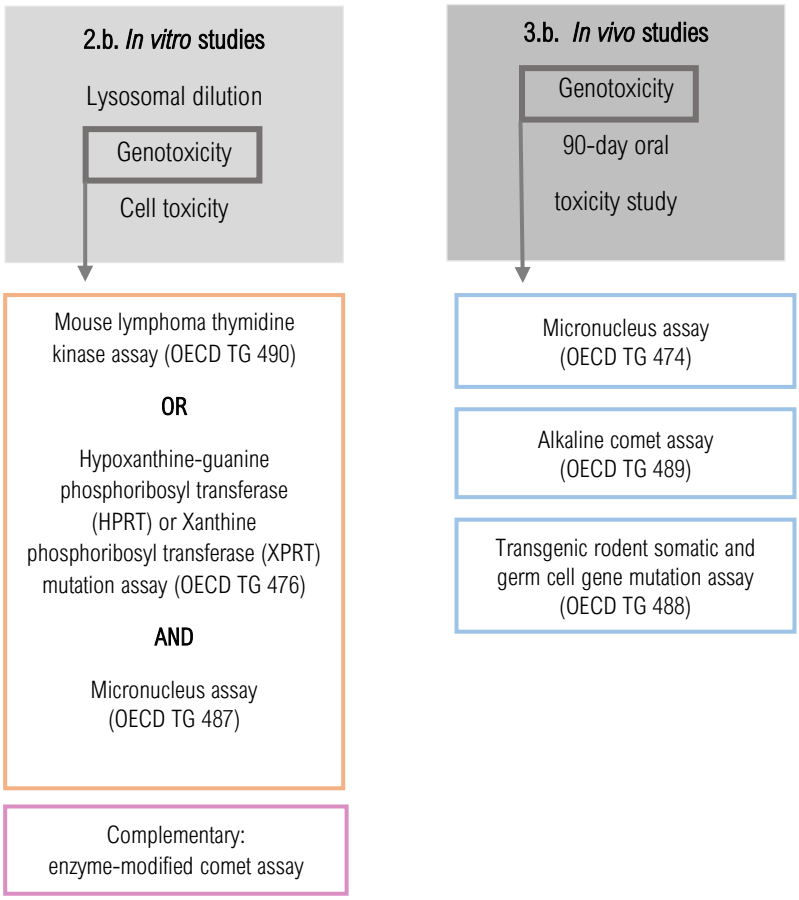

Figure 2. Scheme of the EFSA 2018 strategy for the *in vitro* and *in vivo* genotoxicity evaluation.

The *in vivo* testing is recommended if positive responses are obtained in at least one of the *in vitro* tests indicating genotoxicity, or if any of the *in vitro* assays is not suitable for the evaluation of the material (EFSA 2018) (Figure 2). However, some OECD guidelines for *in vitro* assays, mention that the methods are not fully adapted to the evaluation of NMs (OECD, 2015, 2014b). Thus, it seems not possible to avoid the *in vivo* phase for a correct hazard identification nowadays. Three suitable assays for NMs *in vivo* genotoxicity evaluation are proposed: the *in vivo* micronucleus test, and/or an *in vivo* mammalian alkaline comet assay, and/or a transgenic rodent somatic and germ cell mutation assay, following their corresponding OECD TG 474, OECD TG 489 and OECD TG 488, respectively (OECD, 2016a, 2011, 1997b). The specific *in vivo* strategy for a given NM is not provided by the guidance; it should be decided on a case-by-case, based on the information available and expert opinions.

NMs are known to produce genotoxic effects through primary mechanisms either direct (*i.e*., direct contact with DNA) or indirect (*i.e.*, protein or mitotic spindle interactions, alteration of checkpoint functions, radical oxygen species (ROS) generation, mitochondrial damage or defences inhibition). Moreover, it has to be noticed that some NMs can cause inflammatory effects leading to the generation of ROS, which can potentially trigger secondary genotoxicity that cannot be detected by *in vitro* systems (EFSA Scientific Committee et al., 2018; Magdolenova et al., 2014; Stone et al., 2009).

For this reason, when conducting an *in vivo* evaluation, the EFSA guidance recommends the combination of an *in vivo* comet assay and other tests included in a repeated-dose oral toxicity study, to provide useful information about the secondary genotoxicity mechanisms caused by inflammation (EFSA Scientific Comittee et al., 2018; Evans et al., 2017; Pfuhler et al., 2013).

6. Toxicity of silver nanoparticles

Information about the toxicity of AgNPs is widely available. AgNPs can be absorbed and distributed to different organs through various routes of administration, including inhalation, ingestion, skin contact, and subcutaneous or intravenous injection (reviewed in Ferdous and Nemmar, 2020; Mathur et al., 2018; Xu et al., 2020). After oral exposure, AgNPs have demonstrated to be deposited in the gastrointestinal tract of rodents, from where they are transported to different organs via blood circulation as liver, kidney, lung and brain, being the liver, the major accumulation site followed by spleen and kidneys. Once in contact with the target organs, varied toxic effects have been observed. More detailed information about these aspects can be found in Ferdous and Nemmar, 2020; Mathur et al., 2018; Xu et al., 2020.

AgNPs genotoxicity studies were reviewed as response to the first objective of this thesis, and it was evidenced the AgNPs ability to produce gene mutations, chromosomal aberrations and DNA damage. A great number of studies had been performed *in vitro* and, in less proportion, *in vivo* (see chapter 4, section 1: Bibliographic revision of the genotoxicity of silver nanoparticles). Furthermore, it was highlighted that no-one of the studies followed the strategy for the genotoxic evaluation according to the recommendations suggested by the regulatory agencies, and standard OECD testing guidelines for genotoxicity assays were rarely used. In this regard, the security of any material containing AgNPs needs to be thoroughly assessed.

7. Toxicity of kaolin

Kaolin is a soft and white aluminosilicate mainly composed of kaolinite ($Al_2O_3(SiO_2)_2(H_2O)_2$) ("Kaolin, Al2H4O9Si2 - PubChem,"; United Nations Ennvironmental Programme et al., 2005). Kaolinite structure consists in tetrahedral silica sheets alternated with octahedral alumina sheets (Figure 3 a, b, c).

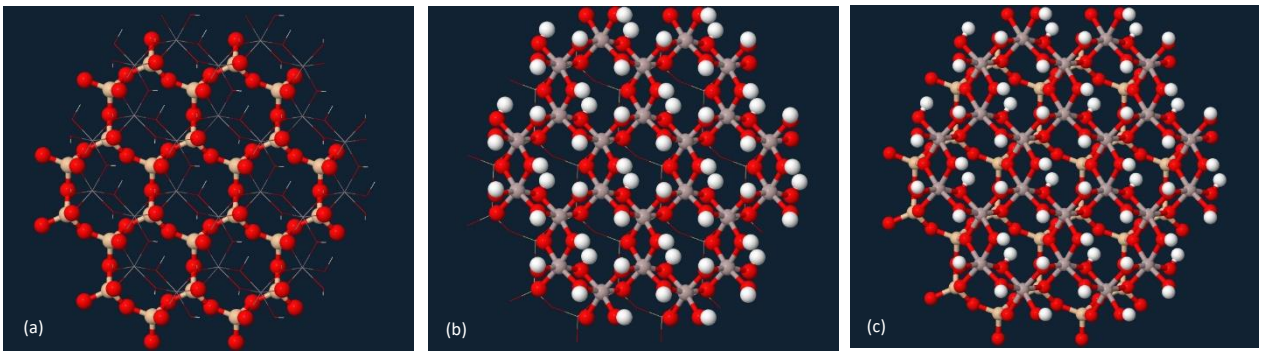

Figure 3. Structure of tetrahedral silica sheets (a), octahedral alumina sheets (b), kaolinite (c). Red spheres: oxygen atom, yellow spheres: silicon atoms, white spheres: hydrogen atoms, purple spheres: aluminium atoms. Obtained from: Kaolinite – Virtual Museum of Molecules and Minerals (wisc.edus).

Kaolin is commonly used in feed additives but, toxicity evaluations are very scarce, old and contradictory (reviewed in Andersen, 2003; Maisanaba et al., 2015). Kaolin is generally considered as an inert material and the toxic effects observed are mainly caused by its over accumulation after administration of high doses. Rats administered with kaolin doses ranging from 1000 to 2100 mg/kg b.w., suffered from listlessness, anorexia, oliguria, hypothermia, and dyspnoea. Those clinical signs were caused by the accumulation of kaolin in the gastrointestinal tract, leading to an overdistension, obstruction and perforation of the small intestine (reviewed in Andersen, 2003). On the contrary, chickens fed with nano-silica platelets at a dose of 1000 mg/kg of feed didn't exhibit concerning changes in hepatic histology, oxidative status, and serum parameters; furthermore, they even increased their intake (Yuan et al., 2017). Some authors reported the dissociation of kaolin in the gastrointestinal lumen and aluminium absorption through the intestinal barrier, while others didn't evidence any measurable absorption (reviewed in Maisanaba et al., 2015).

With respect to genotoxicity, Li and colleagues investigated the safety of a material based on nano-silica platelets by the *in vitro* comet assay, the *in vivo* MN test and the Ames test, obtaining negative results in the three genotoxicity assays (Li et al., 2010). Surprisingly, micro and nano-kaolin particles have been tested in human keratinocytes, human dermal fibroblasts and CHO AA8 cells, and they have produced significant *in vitro* MN increase in a concentration and size dependent manner, with no differences due to cell type (Kawanishi et al., 2020). Micro-kaolin particles also showed increased MN frequencies in A549 cells in a dose-dependent manner and induced DNA damage in the lungs of C57BL/6J mice, measured by comet assay (Totsuka et al., 2009). Both pseudohexagonal and spherical kaolin material induced significant DNA damage in the lungs of ICR mice, being the damaging potency of pseudohexagonal kaolin much stronger than that of spherical ones (Kato et al., 2017).

As previously mentioned, in the last years, several silver-clay based materials have been developed in order to transport silver on an inert vehicle (Abdel-Aziz et al., 2015; Awad et al., 2021; Devi et al., 2014; Hariram et al.,

2021; Hassouna et al., 2017). However, security determinations of clays combined with AgNPs are scarce and genotoxicity studies of materials composed of both AgNPs and kaolin have not been found. In this regard, the safety evaluation of AgNPs-kaolin composites used in or destined for the food industry is of great interest.

Nonetheless, some safety evaluations of other materials composed by different clays as carriers of AgNPs have been conducted, concluding that clays have very low toxicity and that the immobilization of AgNPs onto their surfaces, reduced silver accumulation in tissues and improved the safety of AgNPs, while their antimicrobial activity remained (Shu et al. 2012, Lin et al. 2013). Shu and colleagues (2012) observed that AgNPs immobilized on nano-silica platelets were less cytotoxic than AgNPs on cultured intestinal cells. A year later the *in vivo* evaluation of the same material, showed higher accumulation of Ag in AgNPs-treated chicks than in those treated with AgNPs immobilized on nano-silica platelets (Lin et al., 2013). Moreover, the AgNPs immobilized on nano-silica platelets only produced a mild inflammatory response to the chicks.

Chapter 2. Aim and objectives

One of the objectives of the OUTBIOTICS project was the replacement of antibiotics in livestock production with bactericidal NMs. A formulation based on AgNPs embedded in a kaolin matrix was developed to be used as a feed additive and contribute to decrease the AMR problem. The antimicrobial activity of AgNPs has been widely demonstrated and the silver-kaolin formulation developed showed antimicrobial activity against a wide spectrum of gram-negative and gram-positive bacteria, including multidrug resistant strains (Pérez-Etayo et al. 2021). Once the effectiveness of the material has been demonstrated, it is essential to conduct a safety assessment. Thus, the main objective of the PhD thesis was:

To carry out the genotoxicity evaluation of the silver-kaolin formulation following the strategy suggested by the EFSA in the document "Guidance on risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain: Part 1, human and animal health" (EFSA scientific committee et al. 2018).

In the EFSA document, several *in vitro* and *in vivo* assays are proposed to cover the three critical genotoxic endpoints: gene mutation, structural and numerical chromosomal aberrations. Also, the comet assay -in its standard and modified version- is suggested as a complementary assay that may provide information on the mechanism of genotoxicity and/or the DNA damage by oxidation.

Thus, the specific objectives of this PhD thesis were:

1. Bibliographic revision of the genotoxicity of silver nanoparticles.

2. Evaluation of the *in vitro* genotoxicity of silver-kaolin formulation.
   - 2.1. Adaptation of the *in vitro* methods to the test material
   - 2.2. Study of gene mutations by the MLA, following the principles of the OECD TG 490.
   - 2.3. Study of chromosomal aberrations by the MN test, following the principles of the OECD TG 487.
   - 2.4. Study of DNA strand breaks, alkali labile sites and oxidized DNA by the comet assay.

3. Evaluation of the *in vivo* genotoxicity of silver-kaolin formulation in rats.
   - 3.1. Dose range finding study to establish the maximum dose to be tested in the repeated-dose toxicity study.
   - 3.2. Combined genotoxicity study composed of the MN test (OECD TG 474) and the comet assay (OECD TG 489), integrated in a repeated dose 28-day oral toxicity study (OECD TG 407).

All the assays were performed in the DDUNAV laboratory of Toxicology that has the good laboratory practices (GLPs) accreditation, and all the studies were carried out under GLPs-like conditions.

Chapter 3. Material and methods

1. Bibliographic revision of the genotoxicity of silver nanoparticles; search strategy

A bibliographic search was carried out through the PubMed database ("Home - PubMed - NCBI,") by entering key words in the advanced search builder. These key words were: genotoxicity OR mutagenicity AND silver AND nanoparticles (included in the title/abstract). In this first phase of the selection process, 146 articles were retrieved through the database search. The most recent reviews of relevance regarding AgNPs genotoxicity dated from 2012–2013; therefore, a filter was applied to select articles published since 2013. This resulted in 106 articles to be assessed for eligibility.

In the first phase the articles were selected by reading the abstract and applying the following inclusion and exclusion criteria:

Inclusion criteria:

- The article contains information on *in vitro* or *in vivo* genotoxicity testing of AgNPs.
- The genotoxicity assay(s) included in the article is (are) well established and validated genotoxicity assays (i.e., those included in the EFSA, ISO or ICH guidelines on genotoxicity testing).

Exclusion criteria:

- The article deals with environmental and ecotoxicity studies
- The article deals with gene expression evaluations.

In case of doubt the article was selected for in depth analysis in the second phase; this involved reading the full article. After applying the inclusion and exclusion criteria, 41 articles were selected. Seventeen reviews were retrieved during the bibliographic search; all articles referenced in these reviews were analyzed and included in the tables if they had not already been included and fulfilled the inclusion criteria. This led to the inclusion of two further articles in the selection.

As mentioned above, only articles that referred to well-established genotoxicity assays were selected. The following *in vitro* assays were considered: (1) the *in vitro* mammalian cell gene mutation tests using the thymidine kinase gene (OECD TG 490) (OECD, 2015) or the HPRT and XPRT genes (OECD TG 476)(OECD, 1997a); (2) the *in vitro* mammalian cell MN test (OECD TG 487) (OECD, 2014a); (3) the *in vitro* mammalian chromosome aberration test (OECD TG 473) (OECD, 2016b); and (4) the *in vitro* comet assay (both standard and modified with oxoguanine glycosylase 1 (OGG-1), formamidopyrimidine DNA glycosylase (Fpg) or endonuclease III (Endo-III)). The following *in vivo* assays were included: (1) the mammalian erythrocyte MN test (OECD TG 474) (OECD, 2016c); (2) the *in vivo* mammalian alkaline comet assay (OECD TG 489) (OECD, 2016a); and (3) the mammalian bone marrow chromosome aberration test (OECD TG 475) (OECD, 1997c).

The results of the review were summarized in six tables and data from each *in vitro* or *in vivo* assay were included in each one. The tables were arranged in logical order of genotoxicity assessments, i.e., *in vitro* assays followed by *in vivo* assays. Table 3 contains the *in vitro* MLA results, Table 4 the *in vitro* MN results, Table 5 the *in vitro* comet results (both standard and enzyme-modified), Table 6 the *in vivo* MN results, Table 7 the *in vivo* chromosome aberration results, and Table 8 the *in vivo* comet results (both standard and enzyme-modified). Evaluations of each table were organized according to NPs size, from smallest to largest, and the results included were those provided and interpreted by each author. Negative and positive results were recorded in tables without any information about the "intensity" of positive results.

2. Test compound

Silver-kaolin formulation was provided by Laboratorios ENOSAN, Zaragoza, Spain. Commercial kaolin was treated through a method under patent (ENOSAN), which enables silver (Ag) to be deposited on its surface as AgNPs. The resulting material was a formulation composed by a kaolin matrix containing AgNPs embedded in its surface. The physicochemical characterization of silver-kaolin formulation was conducted by the Group of Analytical Spectroscopy and Sensors of the University of Zaragoza.

The crystalline phase composition of the silver-kaolin formulation was determined by X-ray diffraction, showing a silver content of 0.83 ± 0.04 % (m/m), with a crystal phase composition of kaolinite (68 %), quartz (12 %), illite (13 %), potassic feldspar (6 %) and metallic silver (0.8 %).

The presence, morphology and size distribution of AgNPs was studied by Field Emission Scanning Electron Microscopy. Micrographs obtained by Field Emission Scanning Electron Microscopy showed the laminar structure of kaolinite microparticles decorated with spheroidal silver nanoparticles (Figure 4).

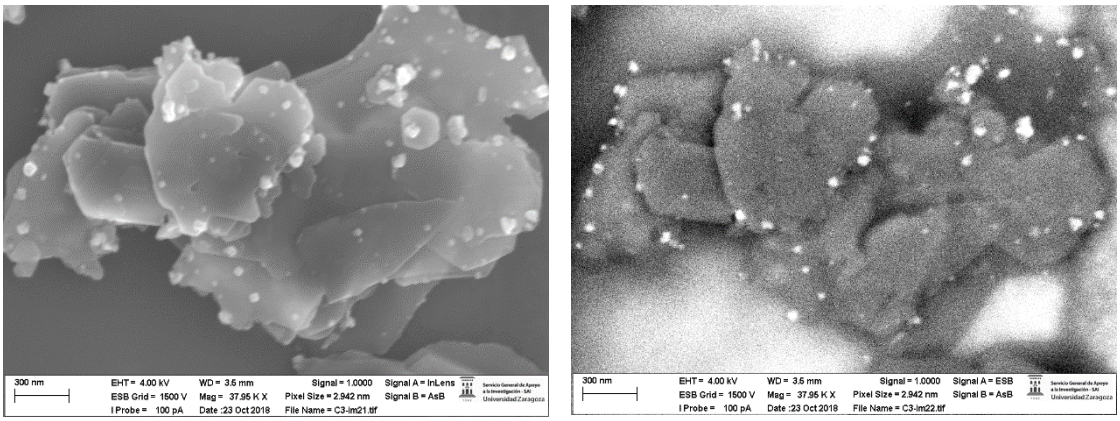

Figure 4. Field emission scanning electron microscopy images of the silver-kaolin formulation.

Silver-kaolin granulometry was studied by X-ray absorption sedimentation. Less than 2 % (m/m) of silver-kaolin microparticles were larger than 25 µm diameter, with ca. 30 % below 1 µm and AgNPs diameters ranged from 2 to 90 nm with an average diameter of 27 nm (Figure 5).

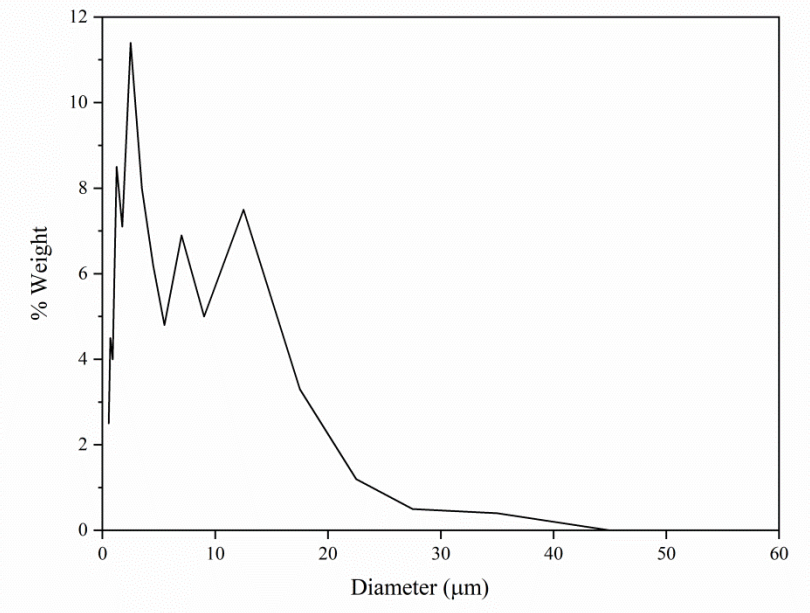

Figure 5. Weight size distribution of the silver-kaolin formulation.

The stability in aqueous suspensions was determined by Dynamic Light Scattering. Aqueous dispersions of the silver-kaolin material showed a sedimentation process over time leaving no particles above 1 µm in suspension after 60 min.

The direct contact of the cells used in the *in vitro* assays with the silver released from the silver-kaolin formulation was evaluated through Single-cell Inductively Coupled Plasma Mass Spectrometry. Cells containing silver were detected by this technique, which implies that silver from the formulation had been internalized or adsorbed by the cells.

Mass distribution obtained by Single Cell Inductively Coupled Plasma Mass Spectrometry analysis showed the silver content (internalized or adsorbed by the cells) corresponding to individual cells, in units of attograms/cell. The technique cannot distinguish between dissolved silver and silver nanoparticles inside the cells (Figure 6).

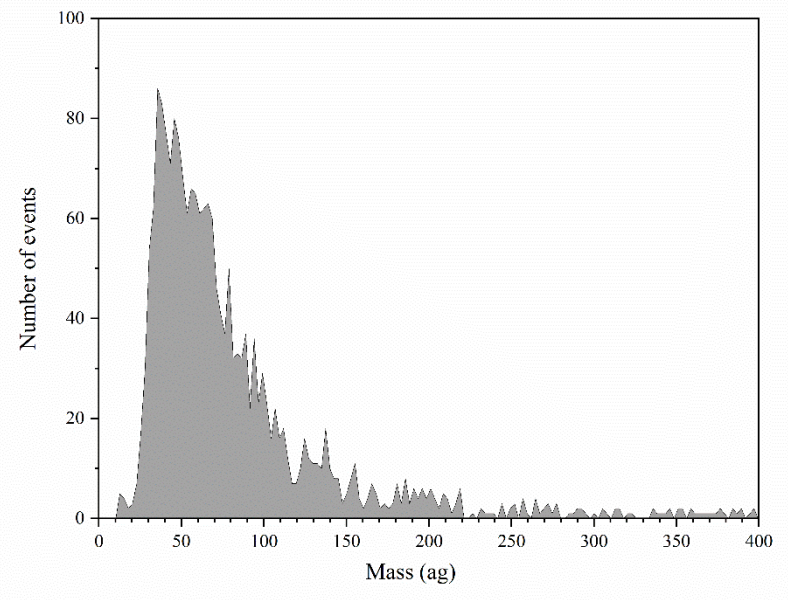

Figure 6. Mass distribution of silver internalized by L5178Y TK$^{+/-}$ cells obtained by Single Cell Inductively Coupled Plasma Mass Spectrometry analysis, after 3 h treatment with 10 mg/mL silver-kaolin formulation.

3. *In vitro* genotoxicity evaluation of silver-kaolin formulation.

With the aim of evaluating the *in vitro* genotoxicity of the silver-kaolin formulation the following experimental design based on the detection of point mutations, chromosome aberrations, and DNA SBs, ALS and oxidation was followed (Figure 7).

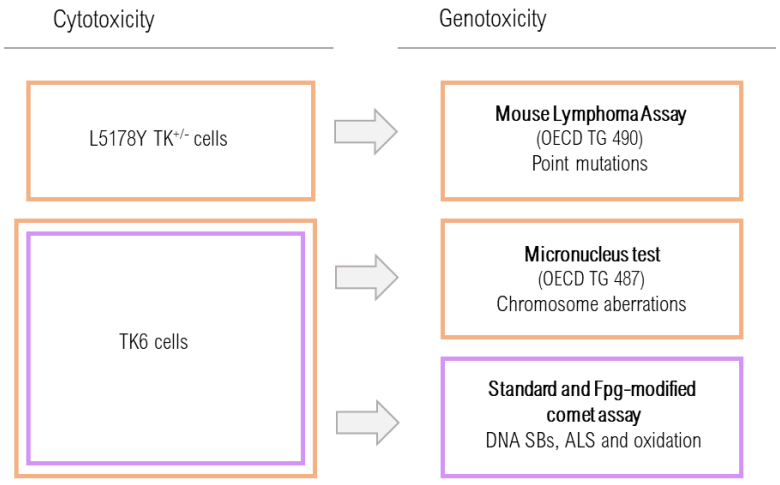

Figure 7. *In vitro* evaluation experimental design.

3.1. Cell lines and cell culture

3.1.1. Chemicals and reagents

RPMI-1640 medium containing D-glucose, HEPES, L-glutamine, sodium bicarbonate and sodium pyruvate (ref. A10491-01), PBS and Pluronic F-68 10 % were purchased from Gibco-Thermo Fisher Scientific. Colchicine, Ethidium Monoazide Bromide (EMA) dye, sytox-green dye, RNAase and beads were obtained from Invitrogen-Thermo Fisher Scientific. Penicillin-streptomycin was purchased from Lonza. Heat inactivated horse serum (HIHS), hypoxanthine, glycine, methotrexate, thymidine, 5-trifluorothymidine (TFT), methyl methane sulfonate (MMS), dimethyl sulfoxide (DMSO) fetal bovine serum (FBS), NaCl, NaOH, KCl, trisodium citrate dihydrate, IGEPAL, sucrose, citric acid, low melting point agarose (LMP), standard agarose, Triton X-100, Tris base, HEPES, Na_2EDTA, BSA and 4,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich. $KBrO_3$, was purchased from Merk. Formamide pyrimidine DNA-glycosylase (Fpg) was provided by NorGenoTech.

3.1.2. L5178Y $TK^{+/-}$ cells

The MLA was conducted using mouse lymphoma L5178Y $TK^{+/-}$ cells, obtained from the American Type Culture Collection [L5178Y $TK^{+/-}$-(clone3.7.2C)] (ATCC® CRL9518™)). Growth medium was composed by RPMI-1640 medium supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, 0.1% Pluronic F-68 and 10 % HIHS (ML10). Medium with the same composition but with 5% or 20% of HIHS (ML5 and ML20, respectively) were also prepared to perform the assay.

Cells were maintained in a humidified atmosphere, using gently shaking at 37ºC and 5% CO_2 at a density of $2x10^5$–$1x10^6$ cells/mL and subcultured for a maximum of two months; then, if needed, new vials were defrosted.

3.1.3. TK6 cells

The MN test and the comet assay were performed in TK6 cells obtained from the ATCC (ATCC® CRL8015™). TK6 growth media was composed by RPMI-1640 medium supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin and 10% FBS.

Cells were maintained in a humidified atmosphere, using gently shaking at 37ºC and 5% CO_2 at a density of $2x10^5$–$1x10^6$ cells/mL and subcultured for a maximum of two months; then, if needed, new vials were defrosted.

3.2. Methodology adaptation; washing procedures

Given the complexity of the material, some of the procedures of the *in vitro* methods were adapted. The *in vitro* assays can be carried out with cells growing in suspension or adherent cells. When soluble compounds are used, they are dissolved in a solvent compatible with cell culture and added to cells. After treatment, if the cells

grow in suspension, the compound elimination consists of a series of washes by centrifugation. When using adherent cells, the washes are usually carried out by removing the cell media containing the treatment and then, by adding and removing new media or buffer several times. Those kinds of washes were not effective for silver-kaolin formulation since it was not soluble in solvents compatible with cell culture. The kaolin-silver formulation was applied to cells in suspension, so alternatives were sought for the removal of the testing compound after treatment.

The assays selected for the *in vitro* evaluation were the MLA, the MN test and the comet assay. Two cell lines derived from lymphocytes were used in the *in vitro* phase, L5178Y $TK^{+/-}$ (MLA) and TK6 cells (MN and comet assays). A maximum concentration of 12 mg/mL of silver-kaolin formulation, was set to carry out the preliminary tests. This concentration was used to determine the most appropriate way to treat and wash the cells in the *in vitro* assays.

Solubility tests were carried out in distilled water and in the cell culture medium and it was found that, as expected, the material was insoluble. When mixing the kaolin-silver formulation with the water or the medium, a suspension of particles of heterogeneous size was obtained, but after thoroughly vortexing for one minute, a more homogeneous suspension of silver-kaolin formulation was obtained.

The cells used for the washes setting up were L5178Y $TK^{+/-}$. Both L5178Y $TK^{+/-}$ and TK6 cells are derived from lymphocytes and grow in suspension, so the results of the methods tested could be applied to both of them. However, it must be considered that the procedures for treating cells in MLA are different than in the MN test and the comet assay. To set-up the cell washing procedures after silver-kaolin formulation treatment, four separation methods were tested.

Differential centrifugation

The first method carried out was the calculation for the separation of cells and silver-kaolin formulation by differential centrifugation; calculations of the centrifuge potency needed to isolate the cells and the silver-kaolin formulation were conducted using the following formula:

$$t = \frac{18\eta}{d^2 \left(p_p - p_m\right) w^2} \ln \frac{x_1}{x_0}$$

"t": time in seconds, "d": viscosity of the medium, "η": density, "w": power, "x_1": final distance of the particle, "x0": distance from the beginning of the container to the beginning of the liquid.

Once determined the power to isolate the cells, it was transformed into r.p.m for the centrifuge through the following formula.

$$w = \frac{2\pi}{60} \text{ r.p.m}$$

Density gradient

L5178Y $TK^{+/-}$ cells are lymphocytes and Lymphoprep™ is an isolation solution for lymphocytes in suspension, commonly used in blood samples. Silver-kaolin formulation was mixed with L5178Y $TK^{+/-}$ cells in suspension at a density of $1x10^6$ cells/mL and then, the mixture was subjected to the lymphocyte separation with Lymphoprep™ following the product protocol: 6 mL of cells and silver-kaolin suspension were mixed with 3 mL of Lymphoprep and centrifuged (800 x g, 20 min) without brake. Then, the interface where lymphocytes must stay was obtained and cells were counted.

Filtration

The sizes of L5178Y $TK^{+/-}$ cells and silver-kaolin formulation were approximately 15 µm (Ngo et al., 2019) and 1 µm (determined by microscope), respectively. A cell strainer was covered with a membrane filter of 5 µm pore diameter. Then, a system based in a 50 mL tube coupled with the cell strainer was prepared. Silver-kaolin formulation particles smaller than the pore would pass through the filter. Silver-kaolin formulation was mixed with L5178Y $TK^{+/-}$ cells at a density of $1x10^6$ cells/mL in suspension and then, the mixture was poured through the cell strainer. The filter content was collected by washing with phosphate buffered saline (PBS) and the cells present were counted.

Decantation

Silver-kaolin formulation was mixed with L5178Y $TK^{+/-}$ cells in suspension at a density of $1x10^6$ cells/mL and then, the mixture was poured in tubes and allowed to decant at different times from 2.5 to 6 minutes. Then, the supernatant was recovered, and cells were counted.

3.3. Test compound preparation

The test compound was assayed at different concentrations in two different forms: 1) the silver-kaolin formulation directly added to the cell cultures, and 2) the silver-kaolin release after 24 hours of continuous agitation of the silver-kaolin formulation in the corresponding cell treatment medium. To this aim, silver-kaolin formulation at the corresponding concentration was added to flasks containing culture medium and maintained in a shaking incubator at 37ºC, 5 % CO_2 for 24 h. Then, flask content was centrifuged (411 x g, 5 min), and the supernatant was recovered to be immediately used for cell treatment.

3.4. Cytotoxicity and proliferation assays

The silver-kaolin formulation cytotoxicity was evaluated just after 3 or 24 h treatment, by measuring the survival of the cells, and proliferation was evaluated 48 h after the treatment by measuring the viability of the cells using the proliferation assays, both with L5178Y $TK^{+/-}$ and TK6 cells. Each proliferation assay consisted in a negative control (i.e., untreated cells) and 5 concentrations of silver-kaolin formulation: a maximum concentration and four serial 1/3 dilutions. Each assay was performed per triplicate.

The maximum concentration tested in L5178Y $TK^{+/-}$ cells for 3 h and 24 h treatment was 10 mg/mL. After treatment, as a measure of cytotoxicity, L5178Y $TK^{+/-}$ cells were counted, compared to the initial cells seeded, and the survival % was calculated for each testing condition in comparison to the negative control. Then, L5178Y $TK^{+/-}$ cells were washed by 5 min decantation and adjusted to $2x10^5$ cells/mL in ML20. After subsequent 24 h incubation cells were readjusted to $2x10^5$ cells/mL in ML20. At last, cells were counted after another 24 h incubation. As a measure of viability 48 h after treatment, total suspension growth (TSG) and relative suspension growth (RSG) % were calculated for each testing condition in comparison to the negative control according to the following formulae.

$$TSG = \frac{\text{total no. cells 48 h after treatment}}{\text{total no. cells before treatment}}$$

$$RSG\ \% = \frac{TSG_{treated}}{TSG_{control}} x100$$

.

The maximum concentration tested in TK6 cells was 5 mg/mL for 3 h and 0.5 mg/mL for 24 h treatment. After treatment, as a measure of cytotoxicity, TK6 cells were counted, compared to the initial cells seeded, and the survival % was calculated for each testing condition in comparison to the negative control. Then, TK6 cells were centrifuged (5 min, 293 x g) to change the culture medium and adjusted to $2x10^5$ cells/mL. After subsequent 24 h of incubation cells were readjusted to $2x10^5$ cells/mL in culture medium. At last, cells were counted after another 24 h incubation. As a measure of viability 48 h after treatment, TSG and RSG % were calculated for each assay condition in comparison to the negative control.

Once the cytotoxicity and proliferation assays after silver-kaolin formulation treatments were carried out, the concentration ranges exerting acceptable levels of cytotoxicity were established for each genotoxicity assay. Then, proliferation assays were carried out, following the same method by testing silver-kaolin release. The maximum silver-kaolin formulation concentration used for silver-kaolin release (see chapter 3, section 3.3. Test compound preparation) and tested in L5178Y $TK^{+/-}$ cells were 10 mg/mL for 3 h and 2 mg/mL for 24 h treatment,

and in TK6 cells were 1.5 mg/mL for 3 h and 0.5 mg/mL for 24 h treatment. Each assay was performed per triplicate. It is worth to mention that more cytotoxicity and proliferation assays were previously carried out, with wider concentration ranges, in order to establish the concentrations tested in these assays.

3.5. Mouse lymphoma assay

3.5.1. Mutant L5178Y $TK^{-/-}$ cleansing

As a preliminary step, to maintain the mutant frequency (MF) within the 50-170x10^{-6} range required by the OECD TG 490, L5178Y $TK^{+/-}$ cells were cleansed to eliminate $Tk^{-/-}$ mutants and therefore, increase the proportion of $Tk^{+/-}$ cells (Fellows Mick D., McDermot Angela, Clare Ketie R., Doherty Ann, 2014; OECD, 2015; Sawyer et al., 1985). A total of 1x10^{7} L5178Y $TK^{+/-}$ cells were grown in THGM medium (variation of ML10 containing 9 µg/mL thymidine, 15 µg/mL hypoxanthine, 22.5 µg/mL glycine and 0.4 µg/mL methotrexate) for 24 h. Then, cells were centrifuged, washed with RPMI medium, resuspended in THG medium (variation of ML10 containing 9 µg/mL thymidine, 15 µg/mL hypoxanthine, 22.5 µg/mL glycine) at a concentration of 1x10^{5} cells/mL, and incubated for 48 h. After 24 h, cells were counted, readjusted with THG medium to 2x10^{5} cells/mL and incubated for further 24 h. Finally, the MF was determined (see sections 3.5.2. Mouse lymphoma assay and 3.5.3. Mouse lymphoma assay calculations), and a stock of cleansed cells was frozen at -140 ºC in aliquots of 1 mL at a concentration of 6x10^{6} cells/mL in ML10 containing 5 % DMSO.

3.5.2. Mouse lymphoma assay

The MLA was carried out following the principles of the OCED TG 490 for microwell version (OECD, 2015). Each experiment consisted in one negative control (i.e., untreated cells), one positive control (i.e., cells treated with 100 µM MMS), and cells treated with 4 concentrations of silver-kaolin formulation or with the corresponding silver-kaolin release (see chapter 3, section 3.3. Test compound preparation). The following concentrations were assayed: 0.12, 0.37, 1.11 and 3.33 mg/mL for short treatment and 0.07, 0.22, 0.67 and 2 mg/mL for long treatment. MMS, an agent which induces point mutations in L5178Y $TK^{+/-}$ cells, was used as positive control, at 100 µM. Each experiment was carried out once.

L5178Y $TK^{+/-}$ cells were treated in continuous agitation for 3 h (short treatment) and 24 h (long treatment) in ML5 and ML10, respectively. For each testing condition, 1x10^{7} cells were exposed to the silver-kaolin formulation or the corresponding silver-kaolin release. For the short treatment, those cells were treated at a concentration of 1x10^{6} cells/mL, in T25 flasks. For the long treatment, the 1x10^{7} cells were exposed to the different treatment conditions at a density of 5x10^{5} cells/mL, in T75 flasks. After treatment, cells were washed by 5 min decantation. Positive control cells were washed twice with PBS by centrifugation (5 min, 293 x g) and seeded in fresh medium.

After washing, cells were diluted to $2x10^5$ cells/mL in 10 mL of ML20 and they were maintained in T25 flasks at 37ºC and 5% CO_2 in a humidified atmosphere, using gently shaking. After 24 h, cells diluted again to $2x10^5$ cells/mL. After another 24 h, two different cell suspensions were prepared per cell culture: one at a density of 10000 cells/mL to score mutant cells, and the second one at a density of 100 cells/mL, to score viable cells. The cells suspension destined to mutant cells scoring was exposed to 400 µg/mL TFT, the selective agent. Both cell suspensions were seeded in 96-well plates: 2000 cells/well in the mutant plates and 2 cells/well in the viability plates. Two identical plates were seeded per condition.

All the plates were then incubated at 37ºC and 5 % CO_2, in a humidified atmosphere, for 10-12 days, until the colonies were formed. Mutated cell colonies (i.e., TFT resistant cells and so $TK^{-/-}$ mutants) were visually counted in the mutant plates and cell colonies were counted in the viability plates. Small and large colonies were counted separately. Small colonies were those covering less than 25 % of the well's diameter and large colonies those covering more than 25 % of the well's diameter (OECD, 2015).

Also, it is worth to mention that as part of this thesis, the Standard Operating Procedure of the MLA was implemented in the Toxicology Laboratory.

3.5.3. Mouse lymphoma assay calculations

Calculations were carried out following the principles of the OECD TG 490 (OECD, 2015). First, cloning efficiency (CE) was calculated for mutant and viability plates as follows.

$$CE = \frac{\left(- \ln \left(\frac{\text{empty wells}}{\text{total wells seeded}} \right) \right) \times \text{total wells seeded}}{\text{seeded cells per well}}$$

Then, to evaluate mutagenicity, the MF was calculated for each assay condition by the application of the following formula, in which "m" is mutant plate and "v" is viability plate.

$$MF = \frac{CEm}{CEv}$$

Then the relative cloning efficiency (RCE) was calculated for each assay condition by the application of the following formula, in which "t" is treatment and "c" is control.

$$RCE = \frac{CEv_t}{CEv_c}$$

Finally, to assess cytotoxicity, the relative total growth (RTG) was calculated for each assay condition by applying the following formula.

$$RTG_{treatment} = \frac{RCE_t x RSG_t}{100}$$

In all cases the mean of the 2 plates of each condition was obtained.

3.6. Micronucleus test

The MN test was carried out following the principles of the OCED TG 487 (OECD, 2014b). Each experiment consisted in a negative control (i.e., untreated cells), and 4 concentrations of silver-kaolin formulation (for long treatment) or 4 concentrations of the corresponding silver-kaolin release (for short and long treatment) (see chapter 3, section 3.3. Test compound preparation). Each experiment also included a positive control (i.e., cells treated with 100 µM of the clastogen MMS, and cells treated with 10 ng/mL of the aneugen colchicine, for the short and the long treatment, respectively).

In the short treatment, $6x10^5$ cells were exposed to each testing condition, for 3 h in a volume of 1 mL in 12-well plates. Afterwards they were centrifuged (5 min, 293 x g) and subcultured for another 21 h (i.e., until 1.5-2 cell cycles). In the long treatment, $3x10^5$ cells were exposed for each testing condition for 1.5-2 cell cycles in a volume of 1 mL in 12-well plates. TK6 cell cycle was previously calculated and 24 h was the time corresponding to 1.5-2 cell cycles. The following concentrations of silver-kaolin were assayed: 0.07, 0.22, 0.67 and 2 mg/mL for 3 h treatment and 0.02, 0.06, 0.17 and 0.5 mg/mL for 24 h treatment. Treatments were carried out in continuous agitation.

After the end of both treatments, cells were centrifuged (8 min, 263 g, 4ºC), then, resuspended in 120 µL of EMA nucleic acid staining (0.025 mg/mL EMA in PBS/FBS 2%) and exposed to a light source (60 W light) on ice for 20 min, which was separated 30 cm from the cells. Cells were then washed with 4 mL of PBS with 2% FBS through centrifugation (8 min, 263 x g, 4ºC). After washing, cells were stained by adding 250 µL of lysis solution 1 (0.2 µM Sytox, 1 mg/mL RNAase, 0.584 mg/mL NaCl, 1 mg/mL trisodium citrate dihydrate, 0.3 µL/mL IGEPAL) and incubated in darkness at room temperature for 1 h. Finally, cells were stained by adding another 250 µL of lysis solution 2 (0.2 µM Sytox, 1.5 µL/mL beads, 85.6 mg/mL sucrose, 15 mg/mL citric acid) and incubated in darkness at room temperature for 30 additional min.

Samples were stored for a maximum of 48 h in darkness at 4ºC. Nuclei and MN were analysed by FACS Canto™ II Six colours (BD, New Jersey, U.S.) by scoring 20000-25000 nucleated cells. MN were gated out following the MicroFlow Instructions Manual from Litron Laboratories (NY, U.S.) by using FlowJoTM V10.2 software (BD, New Jersey, U.S.).

The frequency of MN for each of the samples and controls was referred to 1000 genomic nuclei (N) by applying the following formula.

$$\text{MN/1000 N} = \frac{\text{Number of MN}}{\text{Number of nucleated cells}} \times 1000$$

Cytotoxicity was calculated for each testing condition by adding a known number of beads to each sample in the lysis solution 2. For this purpose, the ratio nuclei/beads was assessed for each sample, compared to the one of the negative control (considering it as the 0% cytotoxicity) and showed as relative survival (RS) %.

The MN/1000 N obtained for each treatment was compared with the negative control through the Chi squared test. Statistical significance was set at $p < 0.05$. In addition, each assay was subjected to a statistical regression test to check if the response obtained was concentration related. Statistical analysis was conducted using Stata/IC 12.1. As MN were assessed by flow cytometry, the MN-fold over the control was also considered in order to evaluate the results.

3.7. Standard and FPG-modified comet assay

The comet assay was performed following the procedure previously described by Collins and Azqueta (2012), with minor modifications. Each experiment consisted in a negative control (i.e., untreated cells), a positive control (i.e., cells treated with 1.25 mM $KBrO_3$) and 5 concentrations of silver-kaolin formulation or 5 concentrations of the corresponding silver-kaolin release (see chapter 3, section 3.3. Test compound preparation). The following concentrations of silver-kaolin formulations were assayed: 0.02, 0.06, 0.17, 0.5 and 1.5 mg/mL for 3 h treatment and 0.01, 0.02, 0.06, 0.17 and 0.5 mg/mL for 24 h. In the short treatment, a total of $6x10^5$ cells were exposed to each testing condition for 3 h in a volume of 1 mL in 12-well plates. In the long treatment, a total of $3x10^5$ cells were exposed to each testing condition for 24 h in a volume of 1 mL in 12-well plates. When cells were exposed to $KBrO_3$, treatment always lasted 3 h. Treatments were carried out in continuous agitation. Each experiment was carried out per triplicate.

After treatment, cells were centrifuged (5 min, 293 x g, 4 ºC), diluted in culture medium to $1x10^6$ cells/mL and mixed with 1% LMP agarose (dissolved in PBS), achieving 0.8% LMP agarose. Two drops of 70 µL of cell suspension per slide were placed on pre-coated slides with 1% standard agarose and a 20x20 mm coverslip

was placed on top of each gel. Three identical slides were prepared for each testing condition. Slides were kept immersed for 1 h in lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, 1% Triton X-100, adjusted to pH 10 with NaOH) at 4ºC. Then, two slides per testing condition were washed with Buffer F (40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/mL BSA, pH 8) three times (5 min each). Afterwards, 45 µL of Buffer F or Fpg enzyme (previously titrated (Muruzabal et al. 2019)) were added on each gel of their corresponding set of slides, and 22x22 mm coverslips were put on top of each gel. Slides were then incubated in a humidified atmosphere, at 37ºC for one hour. After that, the coverslips were removed and all the slides (including the set of slides kept in lysis solution) were immersed in electrophoresis solution (1 mM EDTA, 0.3 M NaOH, pH 13) during 40 min at 4ºC. Finally, slides were subjected to electrophoresis (1 V/cm, 300 mA) for 20 min, at 4 ºC and neutralized by washing them with PBS followed by distilled water (10 min, 4ºC each wash).

Finally, each gel was stained with 30 µL of 1 µg/mL DAPI solution and comets were analysed by fluorescent microscope (Nikon Eclipse 50 i, Japan) using the image analysis system Comet Assay IV (Instem, UK). A total of 100 randomly selected cells were analysed per slide, 50 cells of each duplicate gel and the DNA damage indicator used was the tail DNA intensity (% DNA in tail). The median % DNA in tail of the 50 comets analysed per gel was calculated and then, the mean of both medians of each gel was obtained. The slides which remained immersed in lysis solution were used to assess strand breaks (SBs) and alkali labile sites (ALS). The difference between the % DNA in tail in the slide treated with Fpg and the % DNA in tail in the slide treated with Buffer F was used to calculate the net Fpg-sensitive sites (i.e., oxidized bases).

The mean % DNA in tail of each testing condition was statistically compared with the negative control through the Kruskal-Wallis test. Statistical significance was set at $p < 0.05$. Statistical analysis was conducted using Stata/IC 12.1. Minimum Information for Reporting Comet Assay (MIRCA) recommendations were followed in this study (Møller et al., 2020).

4. *In vivo* toxicity and genotoxicity evaluation of silver-kaolin formulation

With the aim of evaluating the *in vivo* safety of the silver-kaolin formulation, the following experimental design based on the detection chromosome aberrations, and DNA SBs, ALS and oxidation, combined with a 28-day oral toxicity study was followed (Figure 8).

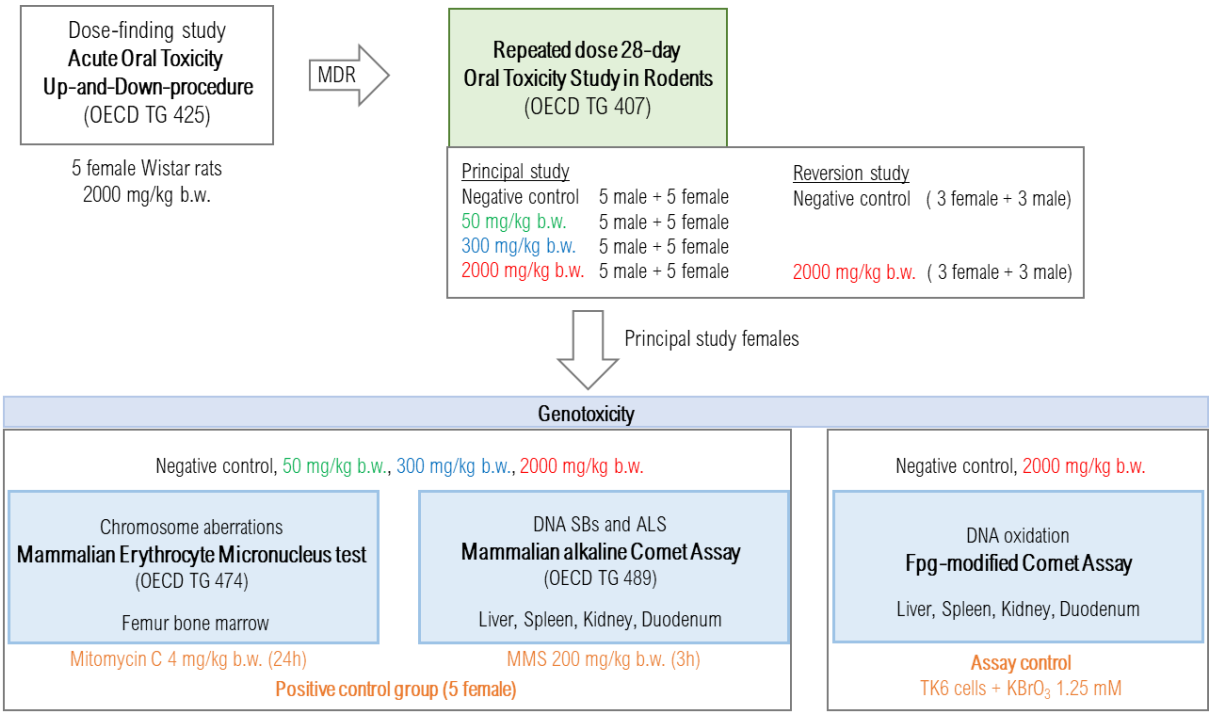

Figure 8. *In vivo* evaluation experimental design.

4.1. Chemicals and reagents

Physiological saline 0.9% and isoflurane (IsoVet®) were purchased from Braun. Sodium citrate dihydrate was purchased from Guinama. Phenylephrine 10 % and tropicamide 1% were purchased from Alcon Laboratories. Penicillin-streptomycin was purchased from Lonza. MMS, FBS, NaCl, trisodium citrate dihydrate, NaOH, KCl, IGEPAL, sucrose, DMSO, citric acid, LMP, standard agarose, Triton X-100, Tris base, HEPES, Na_2EDTA, Hartman´s Fixative (Davidson´s Fixative), BSA, DAPI, giemsa, DPX mounting medium, albumin, aspartate amino transferase (AST), alanine amino transferase (ALT), bilirubin (BIL), brilliant Cresil Blue, cholesterol (CHO), triglycerides (TG), creatine phosphokinase (CPK), creatinine (CREA), alkaline phosphatase (ALP), glucose (GLU), protein, urea, calcium (Ca), potassium (K), precinorm 1, precinomr 2, calibrator, ISE low standard, ISE high standard, ISE reference solution, ISE diluent, ISE internal standard, ECO-D, NaOH, NaCl, cellpack (EPK), stromatolyser-4dl (FFD), stromatolyser-4ds (FFS), sulfolyser (SLS), stromatolyser-fb (FBA) and control blood were purchased from Sysmex. Prothrombin time (PTT-a), fibrinogen, Neoplastin Plus, STA calcium chloride 0.025M, Preciclot Universal I/II, Owren-koller 00360 Limpcub1 and Limpcub 2, were purchased from Sigma. Absolute methanol, formaldehyde 4% and ethanol 96% were purchased from Panreac. Fpg was provided by NorGenoTech, Oslo, Norway. Test strips Control-Test® M and test strips Combur 10 Test® M were purchased from Roche. $KBrO_3$ was purchased from Merk. RPMI-1640 medium containing D-glucose, EMA, HEPES, L-glutamine, sodium bicarbonate and sodium pyruvate (ref. A10491-01), PBS were purchased from Gibco-Thermo Fisher Scientific.

4.2. Animals

Eight-week-old Wistar rats of approximately 160 g for females and 243 g for males (weight variation did not exceed ± 20%) were purchased from ENVIGO. After their arrival, animals were weighted and housed in groups of five in polypropylene cages, at the following environmental conditions (15 air changes/hour, 12 h day/night cycle, 22 ± 2 ºC, relative humidity 55 ± 20%). The animals of the dose finding study and the repeated dose 28-day oral toxicity study (see following sections) were allowed to acclimatize for 5 or 12 days, respectively. Animals were provided with *ad libitum* access to water and controlled access to food.

4.3. Dose finding study

4.3.1. Experimental design

A dose-finding study was carried out following the principles of the OECD TG 425 (OECD, 2008). Silver-kaolin suspensions were freshly prepared each day in distilled water and vortexed for proper mixing. Then, they were administered orally, using a gastrointestinal cannula, at a dose of 2000 mg/kg b.w., in a dosing volume of 1 mL/100 g b.w. A group of five females were used in the study. The first day of the study, the first animal was administered with the first administration. As no lethality was observed after 24 h, the second dose was administered to the first animal and the first dose was administered to the second animal. After confirming the absence of lethality, 24 hours later, the administrations were continued, including the first dose of the third animal, and the second and third doses to the second and first animals, respectively. Finally, 24 h later, the fourth and fifth animals were included in the study. In summary, each of the five animals of the study received 2000 mg/kg b.w. daily, for 7 days, because not lethally was observed. Twenty-four hours after the last dose, the animals were sacrificed in a CO_2 chamber.

4.3.2. Determinations

General symptomatology was deeply observed 30 minutes, 1, 2, 4 and 8 hours after the first administration and daily until the end of the study following the procedure previously described by Irwin (1968).

The day previous to sacrifice, all animals were fasted for approximately 15 hours. Previous to sacrifice, blood samples were obtained, and the following parameters were determined following the same methods as in the repeated dose 28-day oral toxicity study (see section 4.4.3. Analytics):

- Hematology: red blood cell count, white blood cell count, haemoglobin, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin, corpuscular haemoglobin mean concentration and platelets count.
- Biochemistry: albumin, aspartate transaminase, alanine transaminase, alkaline phosphatase, creatinine, total cholesterol, total protein and urea.

Pathology study was carried out following the same methods as for the repeated dose 28-day oral toxicity study (see section 4.4.4. Pathology). The target organs studied were:

- Spleen, heart, liver, kidneys, thymus and ovaries.

The histopathological study was limited to organs with macroscopic and/or weight alterations.

4.3.3. Results evaluation

General symptomatology was compared with the normal values established in the Irwin tests (Irwin, 1968). The mean and standard deviation (SD) of every biochemical and pathological parameter evaluated were obtained. Both the individual data for each animal and the group mean were evaluated. It was verified whether the data obtained were within the reference control values collected in the historical database of the toxicology laboratory. Clinical and behavioural alterations as macroscopic lesions, analytical and pathological alterations, variations in body weight, mortality and other toxic effects, were also compared with the reference control values collected in the historical database of the toxicology laboratory and the data provided by the animal supplier (Envigo) for healthy Wistar rats. Then, if the tested dose did not compromise animal viability, it was identified as the reference dose for the selection of the maximum dose to be tested in subsequent longer duration toxicity studies (Maximum repeated dose, MRD).

4.4. Repeated dose 28-day oral toxicity study

4.4.1. Experimental design

A repeated dose 28-day oral toxicity study in rodents was carried out following the principles of the OECD TG 407 (OECD, 2018a). Fifty-two rats were used in the study (n=26 female and n=26 male). Animals were randomly divided into a) 4 groups of 10 animals each (n=5 female and n=5 male) for the principal study; each group received distilled water (negative control) or, 50 mg/kg (low dose), 300 mg/kg (medium dose) or 2000 mg/kg (high dose) b.w. of the formulation, and b) two groups of 6 animals each (n=3 female and n=3 male) for a potential reversion study; one group received the distilled water (negative control) and the other 2000 mg/kg b.w. of silver-kaolin formulation (high dose).

Fresh silver-kaolin formulation suspensions were daily prepared in distilled water and vortexed for proper mixing. Silver-kaolin formulation suspensions or distilled water in the case of negative control group were administered daily, for 28 days, orally, using a gastrointestinal cannula, in a dosing volume of 1 mL/100 g b.w. Twenty-four hours after the last dose, the animals of the principal study were sacrificed in a CO_2 chamber. Fourteen days after the last dose, the animals of the reversion study were sacrificed in a CO_2 chamber.

4.4.2. Clinical observations

General symptomatology was observed weekly, for 28 days or 42 days for the animals of the principal study or reversion study, respectively, following the procedure previously described by Irwin (1968). Besides, all animals were subjected to a fundus revision after pupil dilatation with an indirect ophthalmoscope (Keeler) and a +78-dioptre lens (Volk), before the first day of administration. The fundus examinations evaluated the response to mydriatics, the transparency of crystalline and vitreous media, the contour and coloration of the optic nerve, the appearance and calibre of retinal veins and arteries, the possible alterations in the retinal parenchyma, lipid or cottony exudates and possible alterations in the retinal pigmented epithelium. The negative control and high dose groups of the principal study were subjected to another fundus revision at the end of the principal study. Body weight and food consumption were monitored weekly until the end of the study.

4.4.3. Analytics

The day previous to sacrifice, all animals were fasted and housed in metabolic cages designed to urine harvest, for approximately 15 hours. Then, blood samples were obtained previous to sacrifice: 1) from the retro orbicular plexus for hematology and serum biochemistry, and 2) from the tail vein for coagulation.

Blood samples for hematology were poured into an $EDTAK_2$ tube. Blood analyses were conducted with an automatic hematologic analyser Sysmex XT-1800i™. The following parameters were evaluated: red blood cell count, white blood cell count, haemoglobin, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin, corpuscular haemoglobin mean concentration, platelets count, differential absolute white blood cells count, differential relative white blood cells count. Also, blood extensions were obtained from each animal and stained with Brilliant Cresyl Blue to perform the reticulocyte count.

Blood samples for serum biochemistry were poured into a separator gel tube and centrifuged (1500 g, 10 min, 20ºC). The serum was separated and analysed with a biochemical autoanalyzer Cobas c-311. The following parameters were evaluated: albumin, aspartate transaminase, alanine transaminase, alkaline phosphatase, creatinine, total cholesterol, total protein, urea total bilirubin, triglycerides, creatine phosphokinase, glucose, sodium, potassium and globulin.

Blood samples for coagulation were poured into a sodium citrate (0.109 M) tube. After centrifugation, serum was obtained and analysed with a coagulometer STart4®. The following parameters were determined: prothrombin time, activated partial thromboplastin time and fibrinogen.

The urine was collected and analysed using Combur 10 Test® M test strips with a Cobas u 411® analyser. The following biochemical parameters were determined: glucose, bilirubin, ketone bodies, density, blood, pH, proteins, urobilinogen, nitrites and leukocytes.

4.4.4. Pathology

After sacrifice, all animals were subjected to an external palpation and detection of wounds or external abnormalities. Then, after opening the abdominal cavity and the thoracic cavity, each target organ was extracted and "in situ" analysed individually, looking for macroscopic abnormalities.

The following tissues were extracted: aorta, spleen, brain, cerebellum, brainstem, heart (atrium and ventricle), oesophagus, stomach, sternum (with bone marrow) and bone marrow smear, femur (with joint), mesenteric nodes, adrenal glands, glands mammary glands (in females), lacrimal glands, salivary glands (submaxillary, sublingual and parotid), liver, pituitary gland, small intestine (duodenum, jejunum and ileum), large intestine (cecum, colon and rectum), tongue, skeletal muscle, sciatic nerve, optic nerve, eyes, pancreas, skin, Peyer's patches, prostate/seminal vesicle, lungs, kidneys, epididymis/uterine tube, testes/ovary, thymus, thyroid/parathyroid, trachea, uterus, cervix-vagina, urinary bladder and medulla spinal. Spleen, brain, heart, liver, adrenal glands, kidneys, prostate/seminal vesicle and testes/epididymis, uterus and ovaries, thymus and thyroid were weighed. Bone marrow smears were prepared from the right femur bone marrow obtained after centrifugation (5 min, 575 g, room temperature). The smears were preserved by wet fixation, using a cytological fixative aerosol. The extracted organs were processed and carved according to the toxicology laboratory protocols. The obtained samples were fixed in formaldehyde 4%, with the exception of the testicles, epididymis and the eyes, that were fixed in Davidson fixative and transferred into a 70% ethanol container after a minimum of 48 h. Finally, each tissue was descaled, carved, included, cut and stained as appropriate to obtain the histological preparations. Then, a complete microscopic evaluation of the preparations was conducted.

4.4.5. Results evaluation

General symptomatology results of the treated groups were compared with the negative control values. The mean and SD of every parameter evaluated were obtained for each sex and group of animals. All data obtained from the weight growth, analytics and organ weights of the principal study groups, were subjected to statistical treatment using the non-parametric Kruskal-Wallis test. If statistically significant differences were found, the results of each group were subjected to statistical treatment using the non-parametric Mann Whitney U test; all data obtained from the principal treated study groups (low, medium and high dose) were compared to those obtained from the negative control group. Statistical analysis was conducted using Stata/IC 12.1. Statistical significance was set up at $p < 0.05$.

In case of finding statistical differences in the mean of treated groups, the individual data of each animal of the group were analysed and compared with historical data of the laboratory.

4.5. Genotoxicity studies experimental design

One extra group of five females was included in the genotoxicity studies as positive control group. The five animals were administered with two positive controls, one for the MN test and other for the standard comet assay. Mitomycin C, an agent that induces MN in bone marrow erythrocytes was used as a positive control for the MN test. MMS, an agent that induces DNA damage in several organs, was used as a positive control for the standard comet assay. A single dose of 4 mg/kg b.w. of Mitomycin C was intraperitoneally administered to each animal in a volume of 1 mL/100 g b.w. 24 h before sacrifice. A single dose of 200 mg/kg b.w. of MMS was orally administered to each animal, through a gastrointestinal cannula, in a volume of 1 mL/100 g b.w. 3 h before sacrifice.

4.6. Genotoxicity tissue collection and sample preparation

In the necropsy, liver, spleen, kidney and duodenum samples were obtained for the comet assay and femur bone marrow samples were obtained for the MN test.

One femur of each animal of the principal groups of the 28-day oral toxicity study was extracted to carry out the MN test. The femurs were sectioned through trochanters and epicondyles, then centrifuged (827g, 5 min, room temperature), the bone marrow obtained, and the extensions prepared. After 10 minutes, the extensions were fixed by introducing them in absolute methanol for 10 min.

Fractions of liver, kidneys, spleen and duodenum were immediately immersed and washed in cold mincing solution (Mg^{++}, Ca^{++} and phenol red-free Hank's balanced salt solution supplemented with 20 mM Na_2EDTA and adjusted to pH 7.5; just prior use 10% of DMSO was added). Sections of approximately 1x1x1 mm, 2x2x2 mm, 2x3x5 mm and 1.5 cm were cut from spleen, liver, kidney (containing both cortex and medulla) and duodenum, respectively. Duodenum sections were rinsed extensively with cold mincing solution and cut open longitudinally, then lightly scraped with a scalpel (1–2 times) and rinsed again with cold mincing solution. Each sample was sectioned multiple times with a round scalpel blade until a mash of single cells was obtained. Then, each sample was dispersed in 1.5 mL cold mincing solution, obtaining cell suspensions. All samples were immersed in ice until processed.

On the other hand, sections of each organ from the negative control and high dose groups were placed in labelled cryotubes, snap frozen in liquid nitrogen and stored at - 80 °C until the Fpg-modified comet assay was

performed. Then, frozen tissue samples were pounded with a tissue crusher (pre-cooled at -80ºC) and dispersed in 1.5 mL of cold mincing solution. All samples were immersed in ice until processed.

4.7. Micronucleus test

The MN test was carried out following the principles of the OECD TG 474 (OECD, 1997b). The evaluation of the induction of MN was carried out on erythrocytes of the femur bone marrow of the females of the principal groups of the 28-day oral toxicity study.

4.7.1. Sample staining and analysis

Once fixed, the bone marrow extensions were introduced in Giemsa 10% in PBS, previously filtered with a Whatman grade 1 filter, in low agitation for 10 min. The dye was then removed from the sample by gently renewing the content of the bucket with tap water for 2-3 min. Finally, the extensions were introduced in a bucket with type II water for 2 min. Then, the samples were dried up on a Whatman filter upside down for 10 seconds and face up during 15 min.

Finally, samples were analysed by eye with an optical microscope, with the x100 objective, using immersion oil. PCE, NCE and MN were scored (see next section).

4.7.2. Results evaluation

To evaluate the haematopoietic toxicity, the rate between the polychromatic erythrocytes (PCE) over the total erythrocytes (in a minimum of 500 normochromic erythrocytes (NCE) and PCE counted), was calculated for each animal. Then, the mean PCE rate of the 5 animals composing each group and their corresponding SDs were obtained. To evaluate the genotoxicity, the MN were determined in at least 4000 PCE and MN % was calculated for each animal, by the application of the following formula.

$$\text{MN \%} = \frac{\text{Number of MN}}{\text{total PCE}} \times 100$$

Then, the mean MN % of the 5 animals composing each group and their corresponding SDs were obtained. The mean MN frequencies and EPC rates of the treatment and negative control groups were statistically evaluated using the non-parametric Kruskal-Wallis test. The mean MN % and EPC rate of the positive control group were statistically evaluated in comparison to the negative control using the non-parametric Mann-Witney U test. Statistical significance was set at $p < 0.05$. Statistical analysis was conducted using Stata/IC 12.1.

4.8. Standard and Fpg-modified comet assay

The standard comet assay was carried out following the principles of the OECD TG 489 (OECD, 2016a). The induction of SBs or ALS was evaluated in fresh liver, spleen, kidney and duodenum samples of the females of the principal groups of the of the 28-day oral toxicity study.

Moreover, the Fpg-modified comet assay was carried out to assess the silver-kaolin formulation induction of oxidized bases. In this case, frozen samples of the aforementioned tissues from animals treated with the highest dose of the formulation (i.e., 2000 mg/Kg) and animals from the negative control group were analysed.

4.8.1. Standard comet assay

Cell suspensions were mixed with 1% LMP agarose (dissolved in PBS), achieving 0.8% LMP agarose. Two drops of 70 µL of cell suspension per slide were placed on pre-coated slides with 1% standard agarose and a 20x20 mm coverslip was placed on top of each gel. Two gels per slide were placed on pre-coated slides with 1% standard agarose. After gel solidification, coverslips were removed, and slides were kept immersed for 1-2 h in lysis buffer at 4ºC. Then, the slides were immersed in electrophoresis solution for 40 min at 4ºC before performing the electrophoresis (1.2 V/cm, 20 min) also at 4 ºC. Finally, slides were neutralized by washing them with PBS followed by distilled water (10 min, each) at 4ºC.

4.8.2. Fpg-modified comet assay

Positive assay controls were included in each assay to assess their correct performance. TK6 cells at a density of 8 x10^5 cells/mL were treated with 1.25 mM $KBrO_3$ for 3 h. After treatment, cells were washed by centrifugation, suspended in growth medium containing a 5 % DMSO, aliquoted and freeze at -80ºC using a Mr. Frosty container. The aliquots were stored at -80ºC until the assay was carried out (no more than 2 months). A vial of positive assay control cells was defrosted for each assay.

Cell suspensions were mixed with 1% LMP agarose (dissolved in PBS), achieving 0.8% LMP agarose. Two drops of 70 µL of cell suspension per slide were placed on pre-coated slides with 1% standard agarose and a 20x20 mm coverslip was placed on top of each gel. Two slides were prepared from each of the four organs of each animal, and from each assay control (one for the Buffer F incubations, see below, and other for the Fpg incubation). After gel solidification, coverslips were removed and slides were kept immersed for 1-2 h in lysis buffer, at 4ºC. Then, the slides were washed with Buffer F three times (5 min each). Afterwards, 45 µL of Fpg enzyme (previously titrated by Muruzabal et al. (2019) or Buffer F were added on the corresponding gels, and 22x22 mm coverslips were put on top of each gel. Slides were then incubated in a humidified atmosphere, at 37ºC for one hour. The Fpg was previously titrated (Muruzabal et al., 2019). Then, the coverslips were removed,

and the slides were immersed in electrophoresis solution for 40 min at 4ºC before performing the electrophoresis (1.2 V/cm, 20 min) also at 4 ºC). Finally, slides were neutralized by washing them with PBS followed by distilled water (10 min each) at 4ºC.

4.8.3. Sample staining and analysis

Gels were stained with 1 mg/mL DAPI solution and comets were analysed by fluorescent microscope (Nikon Eclipse 50 i, Japan), using the image analysis system Comet Assay IV (Perceptive instruments, UK). A total of 150 randomly selected cells were analysed per slide, 75 cells of each duplicate gel, and the DNA damage indicator used was the tail DNA intensity (% DNA in tail). The median % DNA in tail of the 75 comets analysed per gel was calculated and then, the mean of both medians of each slide was obtained. In the case of the standard comet assay, the number of highly damaged comets was determined.

4.8.4. Results evaluation

The mean % DNA in tail and its corresponding SDs were obtained for each organ of the five animals composing each group. In the standard comet assay, the % DNA in tail refers to the presence of SBs or ALS. In the Fpg-comet assay, the net Fpg-sensitive sites, was calculated by the difference between the % DNA in tail in the slide treated with Fpg and the % DNA in tail in the slide treated with Buffer F. The % DNA in tail of each organ of the silver-kaolin formulation treatment and negative control groups were statistically evaluated using the non-parametric Kruskal-Wallis test. The % DNA in tail of each organ of the positive control group, were statistically evaluated in comparison to the negative control group using the non-parametric Mann-Witney U test. Statistical analysis was conducted using Stata/IC 12.1. Statistical significance was set at $p < 0.05$. MIRCA recommendations were followed in these studies (Møller et al., 2020).

Chapter 4. Results

1. Bibliographic revision of silver nanoparticles

A total of 43 articles were selected for data extraction: 31 included *in vitro* studies, 11 included *in vivo* studies and one included both. Although retrieved from the search, two articles that included the comet assay were rejected, due to lack of consistency between the results presented in the figures and the results described.

1.1. *In vitro* studies

In vitro studies were used in almost all articles retrieved (32/41), with the following distribution: the MLA was used in one article (Table 3), the MN assay in 16 articles (Table 4) and the comet assay in 23 articles (Table 5).

With respect to the MLA assay, Guo et al. (2016) treated L5178Y cells (in suspension) with 20, 50 or 100 nm citrate- or PVP-coated AgNPs for four hours. The test was carried out in accordance with OECD TG 490 (OECD, 2015), and the results were evaluated in accordance with the guideline: "Positive responses require a concentration-dependent increase in MF and an induced MF after treatment, in one or more cultures, above the GEF of 126 mutants per 10^6 cells." As shown in Table 3, the results obtained for every NP size and coating were positive.

Table 3. *In vitro* mouse lymphoma assay results.

NP Size (nm)	Coating	Cells	H	µg/mL	Result	Ref.
20	Citrate	L5178Y	4	1–60	+	(Guo et al., 2016)
	PVP				+	
50	Citrate				+	
	PVP				+	
100	Citrate				+	
	PVP				+	

PVP: polyvinylpyrrolidone; H: treatment duration in hours; µg/mL: range of doses tested; +: positive result, according to OECD TG 490 criteria (see text).

The *in vitro* MN assay was used in 16 of the papers selected, with a total of 59 determinations (i.e., assays performed with different cell lines, treatment durations and concentration ranges); 39 of these presented positive results and 20 presented negative results (Table 4).

OECD TG 487 (OECD, 2014a) was followed in studies carried out by five authors: Sahu et al. (2014), Ivask et al. (2015), Guo et al. (2016), Sahu et al. (2016b) and Sahu et al. (2016a). According to the OECD TG 487, human peripheral blood lymphocytes and some rodent cell lines such as CHO, V79 and L5178Y or human cell lines such as TK6 are considered suitable for performing this assay; however, other cell lines such as HT29, Caco-2, HepG2 and A549, which have been used in several studies, have not been extensively validated by the guideline (OECD, 2014a).

Many of the studies in this review have used other human cell lines as experimental models for the MN assay, including: human lymphocyte cell lines (JURKAT, WIL2-NS, TPH-1) by Ivask et al. (2015) and Butler et al. (2015); human bronchial epithelial cells (HBEC) by Lebedová et al. (2018); human breast cell lines (MCF-10A, MCF-7, MDA-MB-231) by Roszak et al. (2017); and human keratinocytes HaCat by Bastos et al. (2017). Some studies detected differences in the sensitivity of cells (Guo et al., 2016; Roszak et al., 2017; Sahu et al., 2016a; Wang et al., 2017).

Table 4. *In vitro* micronucleus assay results.

NP Size (nm)	Coating	Cells	H	µg/mL	Result	Ref.
5		HBEC	48	1–20	-	(Lebedová et al., 2018)
		TK6	28	1–1.5	+	(Y. Li et al., 2017)
10		CHO-K1	24	0.025–2.5	+	(Souza et al., 2016)
		CHO-XRS5			-	
		JURKAT E61	24	1–25	+	(Butler et al., 2015)
		TPH-1			+	
15		MCF-10A	24	5.9–23.5	-	(Roszak et al., 2017)
		MCF-7	48	4.1-16.3	-	
		MDA-MB-231		1.2–4.9	-	
	BSA	CHO-K1	24	1–10	+	(Jiang et al., 2013)
20–50		HepG2	48	12.5–200	+	(Che et al., 2017)
		A549			+	
		HepG2	24	12.5–200	+	(Wang et al., 2017)
		A549			+	
20		JURKAT	24	1–25	+	(Butler et al., 2015)
		TPH-1			+	
		HepG2 (s)	48	5–15	-	(Sahu et al., 2014)
		CACO2(s)			-	
		HepG2 (a)		1–10	+	
		CACO2 (a)			-	
	PVP	HepG2	24	20–160	+	(Wang et al., 2019)
					+	
	Citrate	Lymphocytes	44	0.2–25	+	(Ivask et al., 2015)
	bPEI				+	
	Citrate	JURKAT	24	0.1–25	+	
	bPEI				+	
	Citrate	WIL2-NS			+	
	bPEI				+	
	Citrate	L5178Y	4	1.25–4	+	(Guo et al., 2016)
		TK6		2.5–15	+	
	PVP	L5178Y		1.25–8	+	
		TK6		2.5–30	+	
30	Citrate	HaCat	24	10–40	+	(Bastos et al., 2017)
			48		+	

Size column, samples of NPs of various sizes are expressed as minimum-maximum size; coating column: blank space means no coating, bPEI: branched polyetherimide, BSA: bovine serum albumin; PVP: polyvinylpyrrolidone; H: treatment duration in hours; µg/mL: range of doses tested; -: negative result, +: positive result, (some authors followed OECD TG 487, see text for explanation).

Table 4. *In vitro* micronucleus assay results (continued).

NP Size (nm)	Coating	Cells	H	µg/mL	Result	Ref.
45		MCF-10A	24	5.9–23.5	-	(Roszak et al., 2017)
		MCF-7	48	4.1–16.3	-	
		MDA-MB-231		1.2–4.9	-	
50		HBEC	48	1–20	-	(Lebedová et al., 2018)
		HepG2	4	10–100	-	(Sahu et al., 2016b)
			24	2.5–25	-	
		CACO2	4	10–100	-	
			24	2.5–25	-	
		HepG2	40–44	1–20	+	(Sahu et al., 2016a)
		CACO2			-	
		JURKAT	24	1–50	+	(Butler et al., 2015)
		TPH-1			+	
	Citrate	L5178Y	4	1.25–20	+	(Guo et al., 2016)
		TK6		2.5–120	-	
	PVP	L5178Y		1.25–30	+	
		TK6		2.5–140	-	
90		Balb3T3 A31-1-1	24	0.17–10.60	+	(Choo et al., 2017)
100		CHO-K1	24	0.025–2.5	-	(Souza et al., 2016)
		CHO-XRS5			+	
		JURKAT	24	1–50	+	(Butler et al., 2015)
		TPH-1			+	
	Citrate	L5178Y	4	1.25–35	+	(Guo et al., 2016)
		TK6		2.5–400	+	
	PVP	L5178Y		1.25–50	+	
		TK6		2.5–400	+	

Size column, samples of NPs of various sizes are expressed as minimum-maximum size; coating column: blank space means no coating, PVP: polyvinylpyrrolidone; H: treatment duration in hours; µg/mL: range of doses tested; -: negative result, +: positive result, (some authors followed OECD TG 487, see text for explanation).

With respect to treatment schedule, a short period of 4–6 h with or without metabolic activation, followed by removal of the test chemical and sampling at a time equivalent to about 1.5–2.0 normal cell cycle lengths after the beginning of treatment, and a continuous treatment without metabolic activation for 1.5–2.0 cell cycle length are required by the OECD guideline for a complete evaluation (OECD, 2014a). In the studies reviewed, treatment usually lasted 24 h or 48 h with a few exceptions that lasted 4 h or 72 h (Choo et al., 2017; Guo et al., 2016; Sahu et al., 2016a) (Table 4). Almost all studies tested uncoated AgNPs, except for some that used PVP-, citrate- or branched polyetherimide coated AgNPs (Guo et al., 2016; Ivask et al., 2015; Wang et al., 2019). The size of AgNPs tested varied from 5–100 nm and treatment concentrations ranged from 0.025–400 µg/mL (Table 4).

Regarding evaluation and interpretation of results, providing that all acceptability criteria indicated by the OECD guideline are fulfilled, an *in vitro* MN test is considered clearly positive when at least one of the concentrations tested exhibits a statistically significant increase in MN compared to the negative control, the response is dose-related and any of the results fall outside the distribution of the historical negative control data (OECD, 2014a).

These criteria were applied by Sahu et al. (2014), Ivask et al. (2015), Guo et al. (2016), Sahu et al. (2016b) and Sahu et al. (2016a). In the other studies, results were statistically evaluated by comparing data from treated cells to data from negative controls; P values of <0.05 were considered positive.

The *in vitro* comet assay was used in 23 of the articles selected, with a total of 103 determinations (assays performed with different cell lines, treatment durations and concentration ranges) (Table 5). The different versions were performed and produced positive and negative results; 79 used the standard (ST) version (63+/16−); 15 used the Fpg-modified version (9+/6−); eight used the Endo-III-modified version (6+/2−); and one used the OGG-1-modified version (+). No OECD guidelines exist for the *in vitro* comet assay but, according to this revision, it is the most commonly used *in vitro* assay to assess genotoxicity of AgNPs. This finding is consistent with a previous observation that the *in vitro* comet assay is the most commonly used method for assessing the genotoxicity of NPs (Azqueta and Dusinska, 2015).

Table 5. *In vitro* comet assay results.

NP Size (nm)	Coating	Cells	H	µg/mL	SC	Fpg	Endo-III	OGG-1	Ref.
5		HBEC	48	1–20	+				(Lebedová et al., 2018)
	PEI + PVP	HepG2	24	0.1–1.6	+	+	-		(Ávalos et al., 2015)
		HL-60			+	+	+		
		NHDF			+	+	+		
		HPF			+	+	+		
5–15	PVA	Blood cells	4	1–50 (µM *)	+				(Martinez Paino and Zucolotto, 2015)
		HepG2			+				
5–20	AOT	HepG2	24	1–10	+				(Brkić Ahmed et al., 2017)
	CTAB				+				
	PVP				+				
	BSA				+				
	PLL				+				
10–30		MCF-7	24	5–150	+				(Farah et al., 2016)

Size column, samples of NPs of various sizes are expressed as minimum-maximum size; coating column, blank space means no coating, AOT: sodium bis(2-ethylhexyl)-sulfosuccinate, CTAB: cetyltrimethylammonium bromide, BSA: bovine serum albumin, PLL: poly-L-lysine, PEI: polyetherimide, PVP: polyvinylpyrrolidone; H: treatment duration in hours, nd: no data available; * µM: treatment concentration micromolar; SC: standard comet assay, Fpg: formamidopyrimidine DNA glycosylase modified comet, Endo-III: endonuclease III modified comet, OGG-1: oxoguanine glycosylase 1 modified comet; +: positive result, -: negative result. Results were statistically evaluated by comparing treated cells with untreated control cells; those with $p < 0.05$ at any of the concentrations tested were considered as positive.

Table 5. *In vitro* comet assay results (continued).

NP Size (nm)	Coating	Cells	H	µg/mL	SC	Fpg	Endo-III	OGG-1	Ref.
10		CACO2	24	1–50	-	+			(Vila et al., 2018)
		CHO-K1	24	0.025–2.5	+				(Souza et al., 2016)
		CHO-XRS5			+				
		JURKAT E6-1	24	2.5–20	+				(Butler et al., 2015)
		TPH-1		1–25	+				
	PVP	BEAS-2B	4	10	-				(Gliga et al., 2014)
			24		+				
	Citrate		4		-				
			24		+				
13–60	Citrate	PK15	24	1–75	+				(Milić et al., 2015)
			48		+				
15		MCF-10A	24	5.9–23.5	+	-			(Roszak et al., 2017)
		MCF-7		4.1–16.3	+	-			
		MDA-MB-231		1.2–4.9	-	-			
20–50		HepG2	48	12.5–200	+				(Che et al., 2017)
		A549			+				
		HepG2	24	12.5–200	+				(Wang et al. 2017)
		A549			+				
20		JURKAT E6-1	24	5–25	+				(Butler et al., 2015)
		TPH-1		5–40	+				
	PVP	HepG2		20–160	+				(Wang et al., 2019)
					+				
27		NIH3T3	6	30.1–90.1	+				(Jiravova et al., 2016)
		SVK14		25.4–76.1	-				
30	Citrate	HaCat	24	10–40	+				(Bastos et al., 2017)
			48		+				
35	PVP	Hela	12	1.25–10	+				(Juarez-Moreno et al., 2017)
			24		+				
		MDA-MB-231	12		+				
			24		+				
		MCF-7	12		+				
			24		+				
		HMEC	24h	2.5–5	+				(Castiglioni et al., 2014)
		ECFC			+				
		BMDC	12	0.03–1	+				(Castro-Gamboa et al., 2019)
40		HepG2	24	0.1–6.7	+	+	+		(Ávalos et al., 2015)
		HL-60			+	+	+		
		NHDF			+	+	+		
		HPF			+	-	-		
	Citrate	BEAS-2B	4	10	-				(Gliga et al., 2014)
			24		+				
45		MCF-10A	24	5.9–23.5	-	-			(Roszak et al., 2017)
		MCF-7			-	-			
		MDA-MB-231		2–8.1	-	-			

Size column, samples of NPs of various sizes are expressed as minimum-maximum size; coating column, blank space means no coating, PVP: polyvinylpyrrolidone; H: treatment duration in hours, nd: no data available; SC: standard comet assay, Fpg: formamidopyrimidine DNA glycosylase modified comet, Endo-III: endonuclease III modified comet, OGG-1: oxoguanine glycosylase 1 modified comet; +: positive result, -: negative result. Results were statistically evaluated by comparing treated cells with untreated control cells; those with $p < 0.05$ at any of the concentrations tested were considered as positive.

Table 5. *In vitro* comet assay results (continued).

NP Size (nm)	Coating	Cells	H	µg/mL	SC	Fpg	Endo-III	OGG-1	Ref.
50		HBEC	48	1–20	+				(Lebedová et al., 2018)
		JURKAT E6-1	24	10–50	-				(Butler et al., 2015)
		TPH-1			-				
		BEAS-2B	4	10	-				(Gliga et al., 2014)
			24		+				
56.4		A549	24	10–50	+				(Kukut Hatipoglu et al., 2015)
			48		+				
60		HEK293T	24	10–40	+				(Jiang et al., 2018)
60–105		A549	24	25	-				(Kukut Hatipoglu et al., 2015)
75	Citrate	BEAS-2B	4	10	-				(Gliga et al., 2014)
			24		+				
100		CHO-K1	24	0.025–2.5	+				(Souza et al., 2016)
		CHO-XRS5			+				
	PVP	GM07492	24	0.01–10	+			+	(Franchi et al., 2015)
		JURKAT E6-1	24	10–50	-				(Butler et al., 2015)
		TPH-1			-				
105		NIH3T3	6	1.3	+				(Tomankova et al., 2015)
		SVK14		2.1	+				
		BJ		2.2	+				
131.5		NIH3T3	6	1.4	+				
		SVK14		2.2	+				
		BJ		2.3	+				

Size column, samples of NPs of various sizes are expressed as minimum-maximum size; coating column, blank space means no coating, PVP: polyvinylpyrrolidone; H: treatment duration in hours, nd: no data available; SC: standard comet assay, Fpg: formamidopyrimidine DNA glycosylase modified comet, Endo-III: endonuclease III modified comet, OGG-1: oxoguanine glycosylase 1 modified comet; +: positive result, -: negative result. Results were statistically evaluated by comparing treated cells with untreated control cells; those with $p < 0.05$ at any of the concentrations tested were considered as positive.

A wide variety of cells were used in the *in vitro* comet assay. Treatment duration ranged from 4–48 h and concentrations ranging from 0.025–200 µg/mL. Regarding AgNPs characteristics, uncoated AgNPs and those coated with different chemicals, including branched polyetherimide, poly(vinyl alcohol), sodium bis(2-ethylhexyl)-sulfosuccinate, cetyltrimethylammonium bromide, bovine serum albumin (BSA), poly-L-lysine, PVP and citrate, have been tested, with sizes ranging from 5–131.5 nm (Table 5). In all studies, the results were statistically evaluated by comparing treated cells to untreated control cells; those with p values of <0.05 were considered positive.

Some of the *in vitro* studies evaluated the uptake of AgNPs inside the cells, which is an essential determination to prove that there has been contact between the cell organelles and the AgNPs (Brkić Ahmed et al., 2017; Butler et al., 2015; Che et al., 2017; Franchi et al., 2015; Gliga et al., 2014; Guo et al., 2016; Jiang et al., 2013; Martinez Paino and Zucolotto, 2015; Milić et al., 2015; Sahu et al., 2014; Souza et al., 2016; Vila et al., 2018). Uptake was demonstrated through several methods such as Transmission Electronic Microscopy, Side Scatter Intensity Analysis by Flow Cytometry, Confocal Microscopy, Raman Spectroscopy and Scanning Transmission Electron Microscopy. When AgNPs are internalized, they are usually stored in vesicles in the cytoplasm (Bastos

et al., 2017; Li et al., 2019). There is no evidence that they reach the nucleus but there is a theoretical possibility of direct DNA contact when the nuclear membrane breaks down during mitosis. Moreover, it is worth noting that AgNPs interactions with the cell membrane can also produce toxic effects, mainly due to oxidative stress. Several authors have studied the influence that the release of Ag^+ ions may have on AgNPs genotoxicity; some concluded that their role is negligible, whereas others obtained inconclusive results or indirect evidence of their influence on the AgNPs cytotoxicity and genotoxicity (Butler et al., 2015; Che et al., 2017; Ivask et al., 2015; Jiang et al., 2013).

The influence of AgNPs size on the genotoxic effect has been addressed by some authors, most of whom found a negative correlation: 5, 10 and 20 nm AgNPs are more cytotoxic and genotoxic than analogous 40, 50, 75 and 100 nm AgNPs (Ávalos et al., 2015; Butler et al., 2015; Gliga et al., 2014; Guo et al., 2016; Sahu et al., 2016a). Lebedová et al. (2018) found no clear evidence of a size-dependent effect between 5 and 50 nm AgNPs and Souza et al. (2016) observed a positive correlation; they found that 100 nm AgNPs were more toxic than 10 nm AgNPs. In Tables 2–4, the correlation between AgNPs size and genotoxicity is not evident because the results are expressed only as positive (+) or negative (−) and the dose-response relationship is not reflected. In general, positive results have been obtained in the three *in vitro* assays with AgNPs of different sizes. Lebedová et al. (2018) and Roszak et al. (2017) obtained negative results in the MN assay with HBEC and several human breast cell lines that were not included in the OECD TG 487 as validated or frequently used cells (OECD, 2014a).

With respect to the influence of AgNPs coating, no clear correlation was observed across the studies reviewed (Tables 3–5). Nevertheless, some authors have studied this aspect and obtained different results with coated and uncoated AgNPs. Ivask et al. (2015) found that branched polyetherimide coated AgNPs produced a higher increase in MN than citrate-AgNPs, and suggested that this was due to the fact that branched polyetherimide coated AgNPs present stronger cellular adhesion and internalization. Guo et al. (2016) observed greater effects in citrate-AgNPs than in PVP-AgNPs, although the effect of coating was less important than the effect of size. Brkic et al. (2017) found that intensity of DNA damage measured by comet tail length and intensity, was dependent on AgNPs coating, being poly-L-lysine coated AgNPs and cetyltrimethylammonium bromide coated AgNPs the most and least damaging, respectively. Finally, Wang et al. (2019) observed that uncoated AgNPs caused more DNA damage than PVP-AgNPs in HepG2 cells (comet assay), but the opposite was observed in the MN assay.

1.2. *In vivo* studies

In vivo studies were included in 12 of the 43 articles retrieved, with a total of 102 determinations (in terms of NPs size, coating, animal model, tissue studied, treatment route and duration and sampling time). Thirty-two of

these showed positive results and 69 of them showed negative results. The *in vivo* MN test was used in seven articles, with a total of 28 determinations (17+/11−) (Table 6). The *in vivo* chromosome aberration test was used in three articles, with three determinations (3+) (Table 7). Finally, the *in vivo* comet test was used in seven articles, with 70 determinations performed with the different versions; 42 ST (6+/36−), 24 Fpg-modified (2+/22−), two Endo-III modified (2+) and two OGG-1 modified (2+) (Table 8).

None of the articles that included the *in vivo* MN test referred to OECD TG 474 (OECD, 2016c). Mice, rat and rabbit animal models were used, and MN were analyzed in liver, blood or bone marrow cells. AgNPs were administered orally (p.o.) (13/28) or intravenously (i.v.) (15/28), in either single- or repeat-dose studies (Table 6). All single-dose treatments were administered through the i.v. route, whereas repeated-dose treatments were administered through either the i.v. route for 3 days or the p.o. route for 5, 7 or 28 days (Table 6). Animals were given uncoated AgNPs or PVP-, silicon- or citrate-coated AgNPs, arranging in size range from 5−629 nm. Doses were higher for oral treatments (4−250 mg/kg b.w.) than for intravenous treatments (0.5−25 mg/kg b.w.). In all studies data from treated animals were statistically compared to data from untreated animals, and those with p values of <0.05 were considered positive. The results do not appear to be influenced by NPs size (Table 6). With respect to surface functionalization, uncoated and citrate-AgNPs produced positive results, whereas PVP-AgNPs and silicon-AgNPs produced negative results, except in the study by Wang et al. (2019), who observed that both uncoated and PVP-coated AgNPs administered for 28 days were positive, albeit only at the highest dose (Table 6).

Table 7 shows the results of the *in vivo* chromosome aberration assay, which was used in three of the articles selected. None of the articles analyzed followed OECD TG 475 (OECD, 2014a) chromosome aberrations were analyzed in bone marrow cells of rats treated through the i.v., p.o., or intraperitoneal (i.p.) route for 1, 5 or 28 days, respectively, with uncoated AgNPs measuring 10 nm or 6−629 nm in size. The dosing and sampling times differed in each study, but the results were always positive (Table 7). The results from treated groups were statistically compared to the negative control results and those with p values of <0.05 were considered positive.

Table 8 shows the results of the seven articles that studied the genotoxic effect of AgNPs through the *in vivo* comet assay. Only Asare et al. (2016) followed OECD TG 489 (OECD, 2016a). Different strains of mouse, rat and rabbit were used as experimental models, and comets were analyzed in cells from liver, lung, testis, blood or bone marrow. AgNPs were administered through the i.v. route (single or 3 days) in four studies and through the p.o. route (single, 5, 35 or 45 days) in three studies. Uncoated and PVP-, silicon-, or citrate- coated AgNPs arranging in size from 5−200 nm were administered to the animals. The doses were higher for oral treatments (5−100 mg/kg) than for intravenous treatments (0.5−25 mg/kg).

Table 6. *In vivo* micronucleus assay results.

NPs Size (nm)	Coating	Animal Model	Tissue	Route	Duration	Dose (mg/kg Body Weight)	ST	Result	Ref.
5	PVP	B6C3F1 mouse	Blood	i.v.	Single dose	0.5–20	2 d	-	(Li et al., 2014)
10		Sprague Dawley rat	Bone marrow	p.o.	5 day	5–100	1 d	+	(Patlolla et al., 2015)
20	Citrate	OGG1 −/− KO C57BL/6 mouse	Blood	p.o.	3 day	4	1 d	+	(Nallanthighal et al., 2017b)
					7 day			+	
		C57BL/6 mouse			3 day			+	
					7 day			+	
	Citrate	C57BL/6 mouse	Blood	p.o.	7 day	4	1 d	+	(Nallanthighal et al., 2017a)
							1 w	+	
							2 w	+	
	PVP						1 d	-	
							1 w	-	
							2 w	-	
		Wistar rat	Bone marrow	i.v.	Single dose	5	1 d	+	(Dobrzyńska et al., 2014)
							1 w	+	
							4 w	-	
						10	1 d	+	
							1 w	+	
							4 w	+	
	PVP	ICR mouse	Bone marrow	p.o.	28 day	10–250	1 d	+	(Wang et al., 2019)
								+	
10-80	Silicon	B6C3F1 mouse	Liver	i.v	Single dose	25	2d	-	(Li et al., 2014)
					3 day			-	
200		Wistar rat	Bone marrow	i.v.	Single dose	5	1d	+	(Dobrzyńska et al., 2014)
							1w	+	
							4w	-	
6.3-629		Sprague Dawley rat	Bone marrow	i.v.	Single dose	5	1d	-	(Wen et al., 2017)
15-100	PVP	B6C3F1 mouse	Liver	i.v.	Single dose	25	2d	-	(Li et al., 2014)

Size column, samples of NPs of various sizes are expressed as minimum-maximum size; coating column, blank space means no AgNPs coating, PVP: polyvinylpyrrolidone; OGG1 −/− KO: 8-oxoguanine glycosylase knockout (mouse strain lacking OGG-1); p.o.: oral once a day, i.v.: intravenous, ST: sampling time; d: day post treatment, w: week post treatment; +: positive result, -: negative result. Results from treated groups were statistically compared to negative control results and those with $p < 0.05$ at any of the doses tested were considered as positive.

Table 7. *In vivo* chromosome aberration assay results.

Size (nm)	Coating	Animal Model	Tissue	Route	Duration	Dose (mg/kg Body Weight)	ST	Result	Ref.
10		Sprague Dawley rat	Bone marrow	p.o.	5 day	5–100	1 d	+	(Patlolla et al., 2015)
		Albino rat	Bone marrow	i.p.	28 day	1–4	0 d	+	(El Mahdy et al., 2015)
6.3–629		Sprague Dawley rat	Bone marrow	i.v.	Single dose	5	1 d	+	(Wen et al., 2017)

Size column: samples of NPs of various sizes are expressed as minimum-maximum size; coating column: blank space means no coating; p.o.: oral once a day, i.v.: intravenous, i.p.: intraperitoneal, ST: sampling time; 0d: day of finishing treatment, d: day post treatment; +: positive result, -: negative result. Results from treated groups were statistically compared to negative control results and those with $p < 0.05$ at any of the doses tested were considered as positive.

Table 8. *In vivo* comet assay results.

NPs Size (nm)	Coating	Animal Model	Tissue	Route	Duration	Dose (mg/kg Body Weight)	ST	SC	Fpg	Endo-III	OGG-1	Ref.
5		Swiss albino mouse	Liver	p.o.	Single dose	5–100	3 h	+				(Kant Awasthi et al., 2015)
							1 d	+				
				p.o.w.	35 day	10–20	3 h	+				
8	Citrate	New Zeland white rabbit	Liver	i.v.	Single dose	0.5–5	7 d	+				(Kim et al., 2019)
							28 d	+				
10		Sprague Dawley rat	Bone marrow	p.o.	5 day	5–100	0	+				(Patlolla et al., 2015)
10-80	Silicon	B6C3F1 mouse	Liver	i.v.	3 day	25	3 h	-		+	+	(Li et al., 2014)
20		Wistar rat	Bone marrow	i.v.	Single dose	5	1 d	-				(Dobrzyńska et al., 2014)
							1 w	-				
							4 w	-				
						10	1 d	-				
							1 w	-				
							4 w	-				

Size column, sets of NPs of various sizes are expressed as minimum-maximum size; coating column: blank space means no AgNPs coating, PVP: polyvinylpyrrolidone; OGG-1 −/− KO: 8-oxoguanine glycosylase knockout (mouse strain lacking OGG-1); p.o.: oral once a day, i.v.: intravenous, ST: sampling time; 0: day of finishing treatment, h: hour post treatment, d: day post treatment, dt: day of treatment, w: week post treatment; SC: standard comet assay; Fpg: formamidopyrimidine DNA glycosylase modified comet; Endo-III: endonuclease III modified comet; OGG-1: oxoguanine glycosylase 1 modified comet; +: positive result; -: negative result.. Asare et al. [99] applied the OECD TG 489 criteria (see text for explanation). In other studies, results from treated groups were statistically compared to negative control results and those with $p < 0.05$ at any of the doses tested were considered as positive

Table 8. *In vivo* comet assay results (continued).

NPs Size (nm)	Coating	Animal Model	Tissue	Route	Duration	Dose (mg/kg Body Weight)	ST	SC	Fpg	Endo-III	OGG-1	Ref.
20		OGG1 $^{-/-}$ KO C57BL/6 mouse	Lung	i.v.	Single dose	5	1 d	-	-			(Asare et al., 2016)
							1 w	-	-			
			Liver				1 d	-	-			
							1 w	-	-			
			Testis				1 d	-	-			
							1 w	-	-			
		C57BL/6 mouse	Lung				1 d	-	-			
							1 w	-	-			
			Liver				1 d	-	-			
							1 w	-	-			
			Testis				1 d	-	-			
							1 w	-	-			
90		Wistar rat	Blood	p.o.	45 day	0.5	0	-				(Martins et al., 2017)
			Liver				0	-				
15–100	PVP	B6C3F1 mouse	Liver	i.v.	3 day	25	3 h	-		+	+	(Li et al., 2014)
200		Wistar rat	Bone marrow	i.v.	Single dose	5	1 d	-				(Dobrzyńska et al., 2014)
							1 w	-				
							4 w	-				
		OGG1 −/− KO C57BL/6 mouse	Lung	i.v.	Single dose	5	1 d	-	-			(Asare et al., 2016)
							1 w	+	-			
			Liver				1 d	-	-			
							1 w	-	-			
			Testis				1 d	-	-			
							1 w	-	-			
		C57BL/6 mouse	Lung				1 d	-	-			
							1 w	-	+			
			Liver				1 d	-	-			
							1 w	-	-			
			Testis				1 d	-	-			
							1 w	-	+			

Size column, sets of NPs of various sizes are expressed as minimum-maximum size; coating column: blank space means no AgNPs coating, PVP: polyvinylpyrrolidone; OGG-1 −/− KO: 8-oxoguanine glycosylase knockout (mouse strain lacking OGG-1); p.o.: oral once a day, i.v.: intravenous, ST: sampling time; 0: day of finishing treatment, h: hour post treatment, d: day post treatment, dt: day of treatment, w: week post treatment; SC: standard comet assay; Fpg: formamidopyrimidine DNA glycosylase modified comet; Endo-III: endonuclease III modified comet; OGG-1: oxoguanine glycosylase 1 modified comet; +: positive result; -: negative result.. Asare et al.(2016) applied the OECD TG 489 criteria (see text for explanation). In other studies, results from treated groups were statistically compared to negative control results and those with $p < 0.05$ at any of the doses tested were considered as positive.

According to OECD TG 489 for the *in vivo* comet assay, a result is considered positive if at least one of the test doses exhibits a statistically significant increase compared to the concurrent negative control, the increase is related to dose when evaluated with an appropriate trend test, and any of the results fall outside the distribution of the historical negative control data for a given species, vehicle, route, tissue, and number of administrations (OECD, 2016a). Asare et al. (2016) applied the OECD TG 489 criteria, results from treated groups in the other studies were statistically compared to negative control groups, and those with p values of < 0.05 were considered positive (OECD, 2016a). As shown in Table 6, most of the results were negative.

The biodistribution of AgNPs was studied in only three of the articles retrieved: Li et al. (2014), Martins et al. (2017) and Wen et al. (2017). In these studies, total Ag was determined through Inductively Coupled Plasma Mass Spectrometry, without differentiating between Ag ions and AgNPs. Li et al. (2014) administered a single dose of 15–100 nm PVP-AgNPs or 10–80 nm silicon-AgNPs and detected Ag in peripheral blood and bone marrow tissue, with a much higher concentration in the bone marrow of animals treated with PVP-AgNPs. After three days of treatment, Ag was also found in the liver. Surprisingly, Li et al. (2014) reported negative results in the *in vivo* MN assay when analyzing blood samples (Table 6). When they performed the *in vivo* comet assay with liver samples, they obtained negative results in the standard version but positive results in the OGG-1- and Endo-III-modified comet assays (Table 8). Wen et al. (2017), intravenous administered a single dose of AgNPs (6.3–629 nm) and detected an accumulation of Ag in several tissues, in the following order (highest to lowest): lung > spleen > liver > kidney > thymus > heart. In bone marrow samples from AgNPs- and Ag+-treated animals, the authors observed an increase in both MN and chromosomal aberrations with respect to control animals, but the differences were statistically significant only for chromosomal aberrations (Tables 6 and 7). Martins et al. (2017) orally administered 90 nm AgNPs for 45 days and observed the higher concentration of AgNPs in blood, followed by liver, and a much lower concentration in kidneys; however, they obtained negative results in the standard *in vivo* comet assay in both blood and liver (Table 8).

Considering all of the *in vivo* results, there is no clear evidence that the genotoxic effect of AgNPs is influenced by NPs size or coating; moreover, this aspect was not the specific objective of the *in vivo* studies analyzed. Only Nallanthighal et al. (2017b) observed that citrate-AgNPs exerted a greater genotoxic effect than PVP-AgNPs after seven days of oral administration (Table 6).

2. *In vitro* genotoxicity evaluation of silver-kaolin formulation

2.1. Methods adaptation; washing steps

NMs pose some challenges in the *in vitro* tests. One of them is their elimination after cell treatment. For this reason, several methods were tried in order to wash the cells from the silver-kaolin material after treatment.

Differential centrifugation

Results of the differential centrifugation calculations for L5178Y TK$^{+/-}$ cells and silver-kaolin formulation are shown in Table 9.

Table 9. Results of the differential centrifugation calculations for L5178Y TK$^{+/-}$ and silver-kaolin formulation.

	L5178Y TK$^{+/-}$	Silver-kaolin formulation
Centrifugation time (s)	300	300
Medium viscosity (g/cm*s)	0.0009	0.0009
Particle diameter (cm)	0.0005	0.0001
Particle density (g/cm^3)	1.07	2.6
Medium density (g/cm^3)	1	1
Power (w)	59.13	61.84
X_1 (cm)	11.8	11.8
X_0 (cm)	3.8	3.8
Centrifuge potency (rpm)	**546.65**	**590.53**

The results of the calculated centrifugation potency needed for the cells and the silver-kaolin formulation separation were similar and thus, it was not possible to set differential centrifugation conditions for them.

Density gradient

Results for L5178Y TK$^{+/-}$ cell recovery after their mixture with silver-kaolin formulation and separation by density gradient medium Lymphoprep™ are shown in Table 10.

Table 10. Results of the cell recovery count after separation through density gradient medium.

Silver-kaolin formulation (mg/mL)	Initial cells	Recovered cells	Recovery rate %
0	8000000	5190000	65
12	8000000	0	0

A very low proportion of cells were recovered after the treatment and the method was then considered not adequate.

Filtration

No separation was achieved as silver-kaolin formulation produced the clogging of the filter. Cell counts were not carried out. Thus, this method of separation was immediately discarded.

Decantation

Results for L5178Y TK$^{+/-}$ cell recovery after their mixture with silver-kaolin formulation at 12 mg/mL and decantation of the mixture are shown in Figure 9.

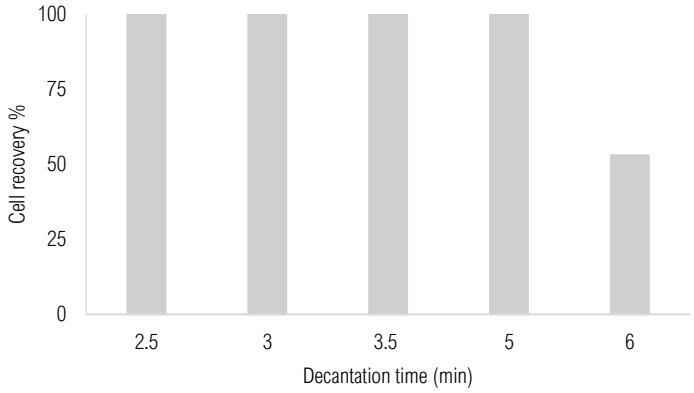

Figure 9. Results of cell recovery after decantation. Each bar represents the % of cells recovered in comparison with the cells initially mixed with the silver-kaolin formulation and thereafter decanted to eliminate as much silver-kaolin formulation as possible.

The percentage of cells recovered after the decantation was 100% in a time range of 2.5-5 minutes. In all cases, a silver-kaolin formulation remnant was observed under the microscope, but it decreased as the decantation time was increased. Thus, decantation for 5 min after cell shaking was considered a good method to recover cells after the treatment. This method was applied in the MLA assay, that is performed in L5178Y TK$^{+/-}$ cells.

Both cell types used in the genotoxicity assays are lymphocytes and grow in suspension, but the methods applied in MLA (L5178Y TK$^{+/-}$) for cell treatment were different than for MN test and comet assay (TK6). In MLA the cell exposure volume was higher than in MN test and comet assay: 5 or 10 mL versus 1 mL. Thus, for the MLA, the method established for silver-kaolin formulation elimination was the decantation of the mixture for five minutes after shaking treatment. Instead, for the MN and comet assays, the washing procedures were carried out by centrifugation.

2.2. Cytotoxicity and proliferation assays

The cytotoxicity of silver-kaolin formulation was evaluated just after 3 or 24 h treatment, by counting the cells, and proliferation was evaluated 48 h after the treatment by calculating the cell growth. The same assays were carried out with the silver-kaolin release. In both cases, three independent experiments were performed for each cell line (L5178Y TK$^{+/-}$ and TK6). The cytotoxicity after the treatment is expressed as survival % and the proliferation 48 h post-treatment as RSG %. Both parameters were considered as affected in values below 80%. Results of the cytotoxicity and proliferation assays on L5178Y TK$^{+/-}$ and TK6 cells are shown in Figure 10 and Figure 11, respectively.

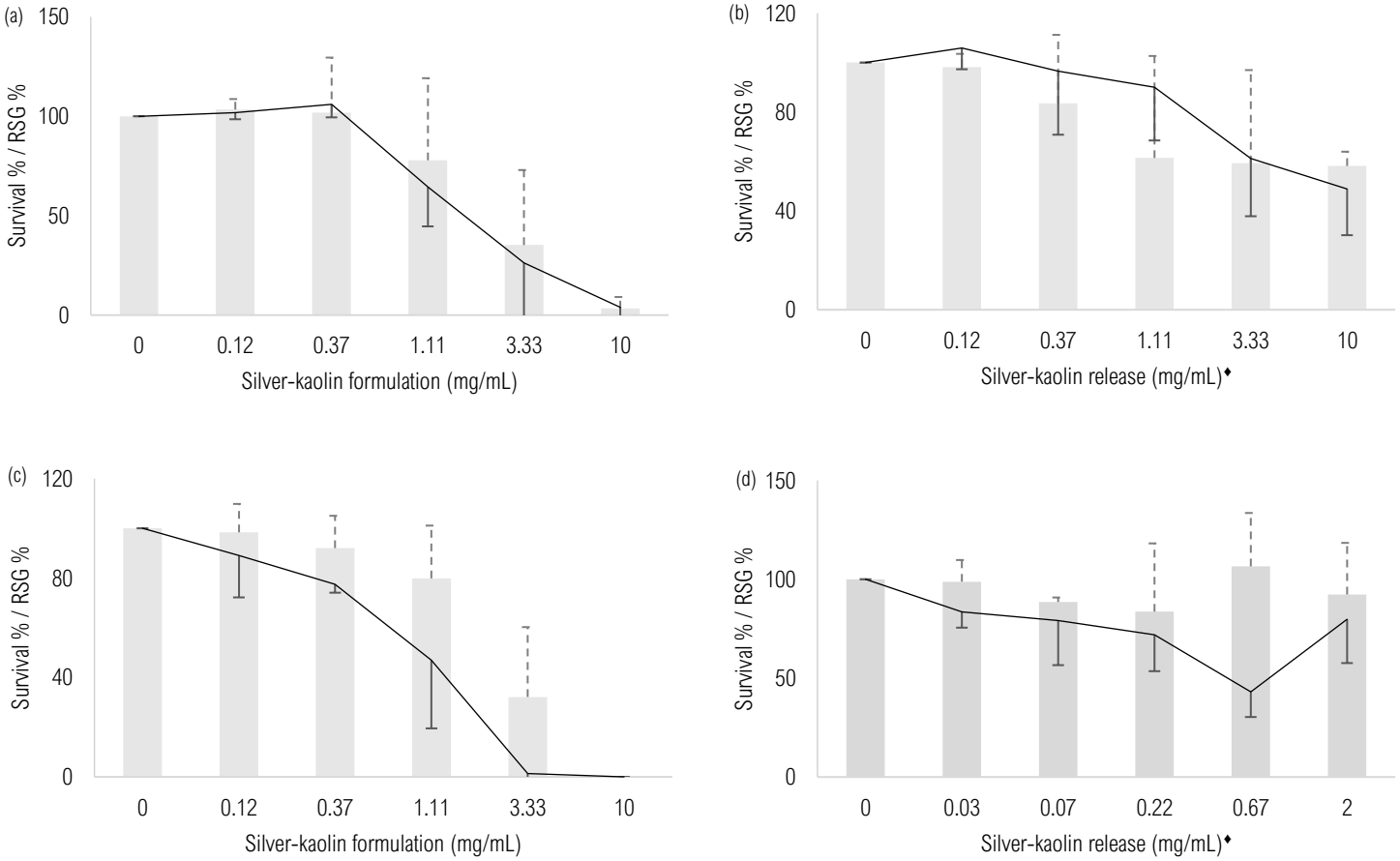

Figure 10. Results of L5178Y TK$^{+/-}$ cells survival %, (bars with positive SD) and RSG % (lines with negative SD). Cells were treated with the silver-kaolin formulation for 3 h (a), silver-kaolin release for 3 h (b), silver-kaolin formulation for 24h (c), and silver-kaolin release for 24 h (d). The means of three independent experiments are represented. ♦ The concentrations indicated correspond to the silver-kaolin formulation that was in agitation for 24h (see chapter 3, section 3.3. Test compound preparation).

According to these results and the principles of the OECD TG 490, the concentrations that were chosen to be tested in the MLA were up to 3.33 mg/mL for 3 h treatment and up to 2 mg/mL for 24 h treatment (OECD, 2015). Three lower 1/3 concentrations (serial dilutions) were also tested.

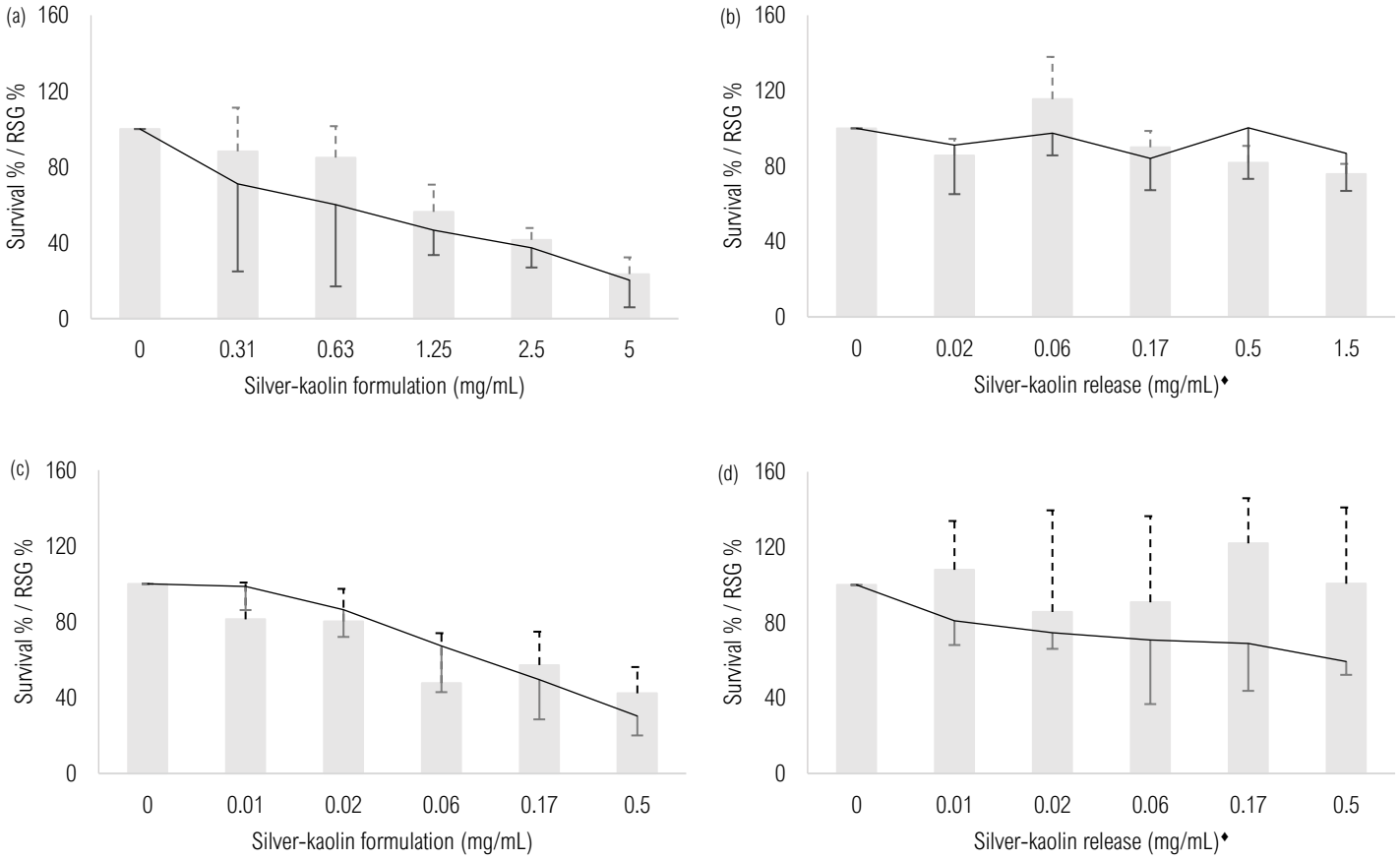

Figure 11. Results of TK6 cells survival %, (bars with positive SD) and RSG % (lines with negative SD). Cells were treated with the silver-kaolin formulation for 3 h (a), silver-kaolin release for 3 h (b), silver-kaolin formulation for 24h (c), and silver-kaolin release for 24 h (d). The means of three independent experiments are represented. ♦ The concentrations indicated correspond to the silver-kaolin formulation that was in agitation for 24h (see chapter 3, section 3.3. Test compound preparation).

For the MN test, following the principles of the OECD TG 487, the concentrations that were decided were up to 2 mg/mL for 3 h treatment and up to 0.5 mg/mL for 24 h treatment (OECD, 2014a). Three lower 1/3 concentrations (serial dilutions) were also tested.

Finally, for the comet assay, the concentrations tested were up to 1.5 mg/mL for 3 h treatment and 0.5 mg/mL for 24 h treatment; and four lower 1/3 concentrations (serial dilutions).

2.3. Mouse lymphoma assay

The induction of gene mutations was assessed by the MLA, in L5178Y TK$^{+/-}$ cells, following the principles of the OECD TG 490 (OECD, 2015). L5178Y TK$^{+/-}$ cells were treated with silver-kaolin formulation and its corresponding silver-kaolin release for 3 h and 24 h treatment. One MLA assay was carried out for each treatment time. Results are shown in Figure 12.

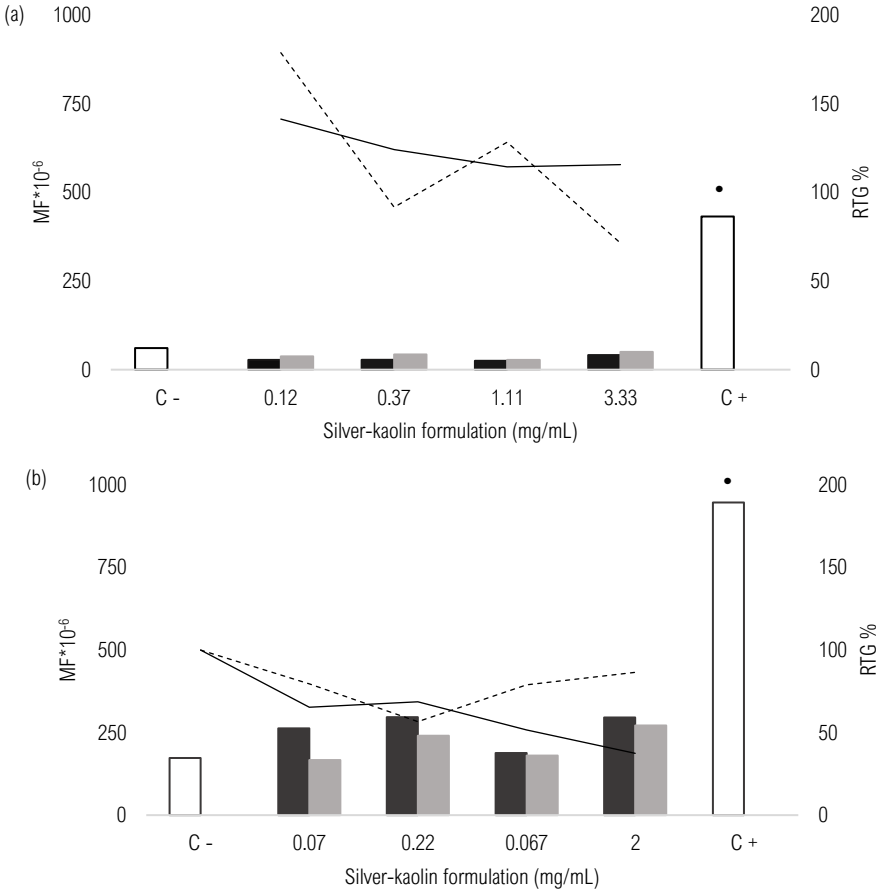

Figure 12. Results of the MLA after 3h (a) and 24h (b) treatment of L5178Y TK$^{+/-}$ cells. Each figure shows the induction of gene mutations, represented as MF *10^{-6}, by silver-kaolin formulation (black bars), its corresponding silver-kaolin release (grey bars), and for the negative (C-) and positive controls (C+) (white bars with black edge). It also shows the cytotoxicity of silver-kaolin formulation (continuous line) and its corresponding silver-kaolin release (dotted line), represented as RTG %. C+: cells treated with 100 µM MMS. •: difference from negative control based on OECD TG 490 GEF. In both graphics, the concentrations indicated correspond to the silver-kaolin formulation added directly to the cell cultures and to the silver-kaolin formulation that was in agitation for 24h (see chapter 3, section 3.3. Test compound preparation).

The silver-kaolin formulation or its corresponding silver-kaolin release didn't produce a MF increase above the negative control at any treatment conditions, thus the material gave a clear negative response. Furthermore, concentration-related effect was not observed. Given the negative results, colonies size was not considered.

2.4. Micronucleus test

The induction of chromosomal aberrations was assessed by MN test, in TK6 cells, following the principles of the OECD TG 487 (OECD, 2014a). TK6 cells were treated with silver-kaolin formulation for 24 h and its corresponding silver-kaolin release for 3 h and 24 h. Two independent MN tests were carried out for each test compound form and treatment time. Results are shown in Table 11.

Table 11. Results of the MN tests. The results of two independent experiments are represented in each case as MN/1000 N and RS %. Statistically significant difference from negative control *: (P< 0.05), **: (P< 0.01).

	TEST 1		TEST 2	
Silver-kaolin release♦ (mg/mL) 3 h treatment	MN/1000 N	RS %	MN/1000 N	RS %
0	1.18	100	0.49	100
0.07	0.94	112	0.29	87
0.22	1.15	93	0.79	97
0.67	0.93	103	0.63	89
2	1.64	128	0.75	67
MMS 150 µM	16.77 **	38	7.53 **	47
Silver-kaolin formulation (mg/mL) 24 h treatment	MN/1000 N	RS %	MN/1000 N	RS %
0	1.71	100	3.40	100
0.02	3.65 *	85	4.60	177
0.05	4.07 *	93	3.21	109
0.17	3.21*	90	7.35 **	133
0.5	1.30	85	7.21 **	65
Colchicine 10 ng/mL	55.25 **	38	78.40 **	74
Silver-kaolin release♦ (mg/mL) 24 h treatment	MN/1000 N	RS %	MN/1000 N	RS %
0	3.74	100	1.73	100
0.02	3.34	102	4.36 **	89
0.05	5.52 **	93	1.27	75
0.17	3.44	81	1.32	77
0.5	2.64	93	2.25	73
Colchicine 10 ng/mL	42.28 **	59	95.90 **	23

♦ The concentrations indicated correspond to the silver-kaolin formulation that was in agitation for 24h (see chapter 3, section 3.3. Test compound preparation)

Silver-kaolin formulation produced some statistically significant MN increase above the negative control, but no testing condition reported an increase of at least 3x MN fold compared to the negative control. Furthermore, no concentration-related effect was observed through the regression test in any of the MN tests carried out. Therefore, the material showed a negative response.

2.5. Standard and FPG-modified comet assay

The induction of SBs and oxidized bases was assessed by the standard and the Fpg-modified comet assay, in TK6 cells, following the procedure previously described by (Collins and Azqueta, 2012). Cells were exposed to silver-kaolin formulation and its corresponding silver-kaolin release, for 3 h and 24 h. Results are shown in Figure 13.

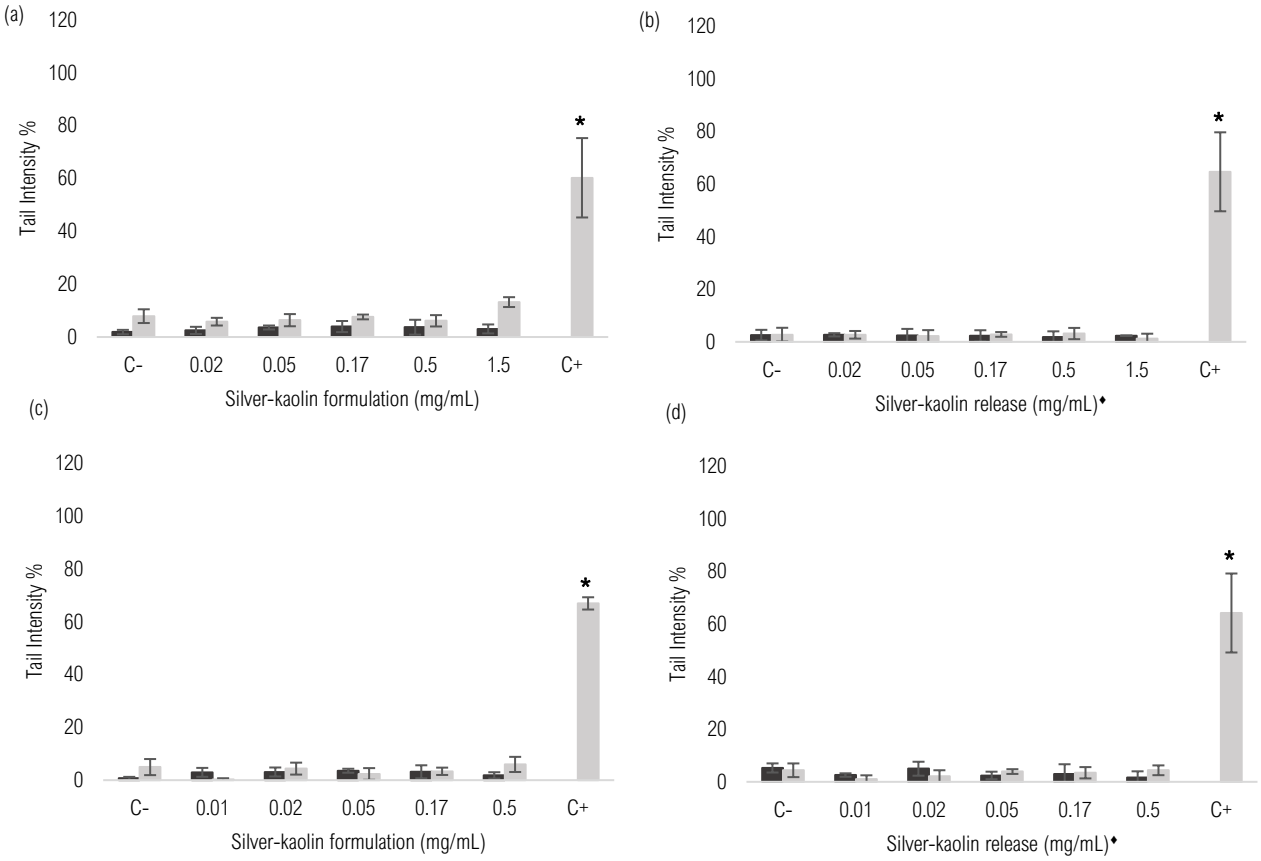

Figure 13. Results of comet assays after 3 h treatment with silver-kaolin formulation (a), 3 h treatment with the corresponding silver-kaolin release (b), 24 h treatment with silver-kaolin formulation (c) and 24 h treatment with the corresponding silver-kaolin release (d). Each figure shows the induction of DNA damage as SBs (black bars) and oxidized bases (Fpg-sensitive sites, grey bars) in TK6 cells, represented as % DNA in tail. Positive control (C+) treatment: cells treated with 1.25 mM $KBrO_3$ for 3 h. The results of three independent experiments are represented in each case as the mean ± the SD. ♦: statistically significant difference from negative control ($P < 0.05$). * The concentrations indicated correspond to the silver-kaolin formulation that was in agitation for 24h (see chapter 3, section 3.3. Test compound preparation).

Neither of the silver-kaolin formulation or its corresponding silver-kaolin release produced statistically significant % DNA in tail increase for ALS, SBs or Fpg-sensitive sites, compared to the negative control, at any treatment conditions. Furthermore, concentration-related effect was not observed. Therefore, the material showed a negative response.

3. *In vivo* toxicity and genotoxicity evaluation of silver-kaolin formulation

3.1. Dose finding study

The clinical signs of the animals did not show alterations throughout the entire study (data not shown). The weight gain of the animals throughout the study (Figure 14) was lower than expected in comparison to the data provided by the animal supplier (Envigo).

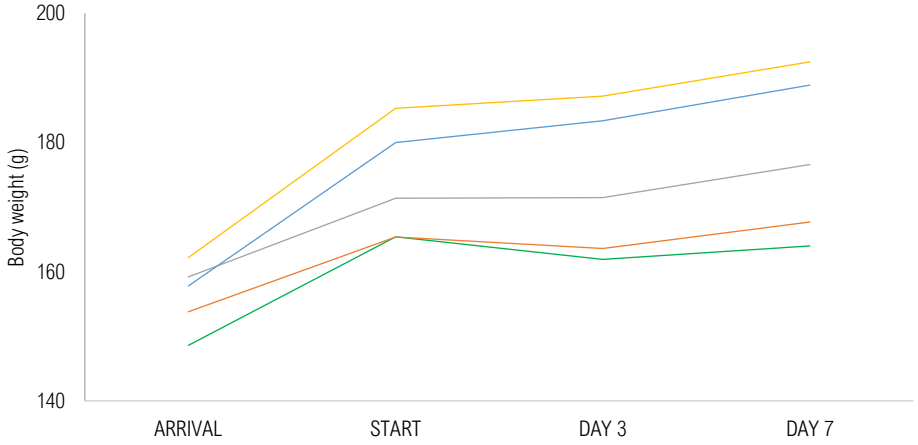

Figure 14. Results of the dose-finding study weight growth. The figure shows the weight growth recording for each animal, expressed as body weight (g) during the 7 days of the study represented in lines (F1: green, F2: orange, F3: grey, F4: yellow, F5: blue). Acclimatation period between arrival and start lasted 5 days. Start: first day of administration.

All animals were sacrificed according to the schedule and no significant macroscopic alterations were found in the organs examined and their absolute or relative weight (Tables 11 and 12), therefore the histological study was not carried out. The hematological parameters evaluated in the five animals after 7 administrations of silver-kaolin formulation, at a dose of 2000 mg/kg b.w., did not show relevant alterations (Table 13). Although some individual values of the biochemical parameters were outside the laboratory records, the group mean was within normal values for all parameters determined (Table 14).

In summary, all animals survived the 7-day of daily exposure of a 2000 mg/kg b.w. dose of silver-kaolin formulation without showing relevant clinical signs. Therefore, 2000 mg/kg b.w. was identified as the maximum dose to be tested in subsequent toxicity assays of longer duration (Maximum Repeatable Dose, MRD).

Table 12. Results of the absolute organ weight for each animal of the dose-finding study (F1-F5). The table collects the individual weights (g) of each organ for each animal and the descriptive statistics of the whole group expressed as mean ± SD. The normality ranges of each parameter (bold letter) were calculated from the laboratory histories (n = 57 animals). •: values outside the laboratory records.

Animal ID	Spleen	Heart	Liver	Thymus	Right kidney	Left kidney	Kidneys sum	Right ovary	Left ovary	Ovaries sum
	0.263-0.592	**0.422-0.945**	**5.068-8.498**	**0.135-0.568**			**1.090-1.808**			**0.060-0.190**
F1	0.350	0.564	4.760•	0.361	0.579	0.584	1.163	0.029	0.042	0.071
F2	0.390	0.498	4.792•	0.401	0.557	0.529	1.086	0.034	0.033	0.067
F3	0.400	0.534	5.710	0.447	0.702	0.673	1.375	0.065	0.069	0.134
F4	0.483	0.592	6.879	0.541	0.761	0.659	1.420	0.064	0.053	0.117
F5	0.411	0.569	5.348	0.562	0.700	0.702	1.402	0.075	0.066	0.141
Mean ± SD	0.407 ± 0.048	0.551 ± 0.036	5.498 ± 0.869	0.462 ± 0.087	0.660 ± 0.088	0.629 ± 0.071	1.289 ± 0.154	0.053 ± 0.021	0.053 ± 0.015	0.106± 0.035

Table 13. Results of the relative organ weight for each animal of the dose-finding study (F1-F5). The table collects the individual weights (g) of each organ for each animal and the descriptive statistics of the whole group expressed as mean ± SD. The normality ranges of each parameter (bold letter) were calculated from the laboratory histories (n = 57 animals). •: values outside the laboratory records.

Animal ID	Spleen	Heart	Liver	Thymus	Right kidney	Left kidney	Kidneys sum	Right ovary	Left ovary	Ovaries sum
	0.138-0.289	**0.198-0.489**	**2.651-4.128**	**0.069-0.285**			**0.546-0.906**			**0.030-0.096**
F1	0.223	0.360	3.038	0.230	0.369	0.373	0.742	0.019	0.027	0.045
F2	0.247	0.316	3.039	0.254	0.353	0.335	0.689	0.022	0.021	0.042
F3	0.240	0.321	3.431	0.269	0.422	0.404	0.826	0.039	0.041	0.081
F4	0.261	0.320	3.718	0.292•	0.411	0.356	0.768	0.035	0.029	0.063
F5	0.231	0.320	3.011	0.316•	0.394	0.395	0.789	0.042	0.037	0.079
Mean ± SD	0.241± 0.015	0.327 ± 0.018	3.247 ± 0.316	0.272 ± 0.033	0.390 ± 0.029	0.373 ± 0.028	0.763 ± 0.052	0.031 ± 0.011	0.031 ± 0.008	0.062± 0.018

Table 14. Results of the hematological parameters for each animal of the dose-finding study (F1-F5). The table collects the individual values of each parameter determined for each animal and the descriptive statistics of the whole group expressed as mean ± SD. The normality ranges of each parameter (bold letter) were calculated from the laboratory histories (n = 240 animals).

Animal ID	RBC ($x10^6$cel/µl)	WBC ($x10^3$cel/ µl)	Haemoglobin (g/dl)	Htc (%)	MCV (fl)	MCH (pg)	CHMC (g/dl)	Platelets ($x10^3$cel/ µl)
	7.53-9.73	**3.51-12.05**	**14.5-17.5**	**41.3-50.1**	**46.2-59.8**	**16.9-20.3**	**32.8-37.2**	**582-1203**
F1	8.52	3.73	15.5	43.9	51.5	18.2	35.3	844
F2	8.68	9.42	15.9	43.9	50.6	18.3	36.2	843
F3	8.05	8.64	15.8	45.8	56.9	19.6	34.5	839
F4	8.07	6.24	15.3	43.3	53.7	19.0	35.3	673
F5	8.31	8.28	15.7	44.4	53.4	18.9	35.4	861
Mean ± SD	8.33±0.28	7.26±2.3	15.6±0.2	44.3±0.9	53.2±2.4	18.8±.06	35.3±0.6	812±78

(): measurement unit. RBC: red blood cell count, WBC: white blood cell count, Htc: hematocrit, MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, CHMC: corpuscular hemoglobin mean concentration.

Table 15. Results of the biochemical evaluation for each animal of the dose-finding study (F1-F5). The table collects the group mean and SD of each parameter evaluated. Normality ranges (bold letter) were calculated from the laboratory histories (n = 240 animals). •: values outside the laboratory records.

Animal ID	Albumin (g/dL)	AST (U/L)	ALT (U/L)	ALP (U/L)	Cholesterol (mg/dL)	Creatinine (mg/dL)	Total protein (g/dL)	Urea (mg/dL)
	4,0-5,3	**39-138**	**10-53**	**13-138**	**43-109**	**0.22-0.56**	**5.4-7.0**	**26-59**
F1	4.1	247•	34	110	59	0.38	5.5	34
F2	4.1	74	15	104	54	0.30	5.4	39
F3	4.6	99	22	93	88	0.31	6.0	40
F4	4.2	64	19	94	98	0.33	5.8	34
F5	4.3	193•	32	104	61	0.36	6.0	32
Mean ± SD	4.3±0.2	135±81	24±8	101±7	72±20	0.34±0.03	5.7±0.3	36±3

(): measurement unit. AST: aspartate transaminase, ALT: alanine transaminase, ALP: alkaline phosphatase.

3.2. Repeated dose 28-day oral toxicity study

All animals survived the 28-day exposure and were sacrificed according to the schedule: day 28 for the animals of the main study and day 42 for the animals of the reversion study. The clinical sings of the animals did not show alterations throughout the entire study (data not shown).

Weight rate gain (Figure 15) and food consumption (Figure 16) were also within the normal limits established by the animal supplier (Envigo) in all the groups and for both males and females. All the ocular structures evaluated in the fundus examinations (i.e., retina, macula, blood vessels, optic nerves, and choroid) as well as the appearance of the media, were found to be normal. Due to the absence of toxic effects in ophthalmological examinations of the negative control and 2000 mg/kg b.w. groups of the principal study, the examinations of the 50 mg/kg b.w., 300 mg/kg b.w. groups were not carried out.

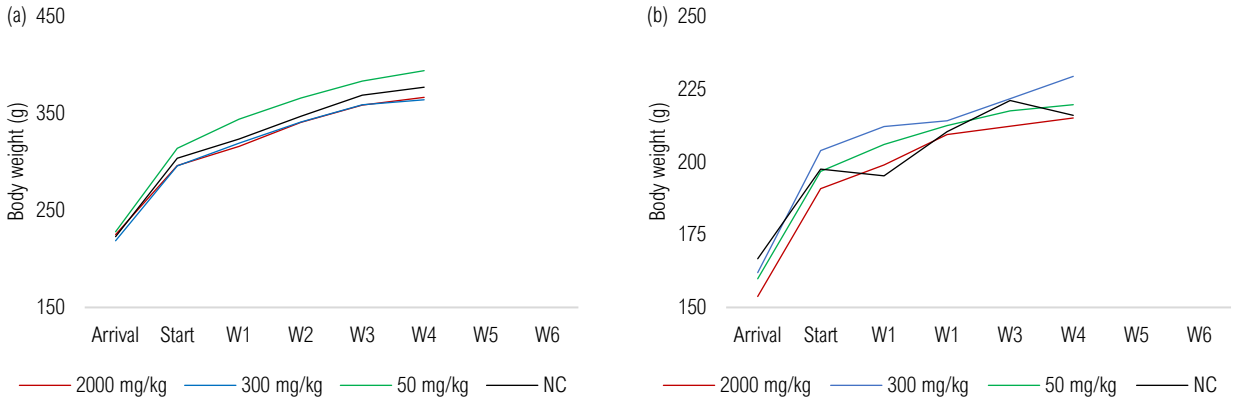

Figure 15. Results of the repeated dose 28-day oral toxicity study body weight growth of males (a) and females (b). Each Figure shows the mean body weight (g), for each study group, throughout the weeks (W) of the study. Acclimatation period between arrival and start lasted 12 days. Start: first day of administration.

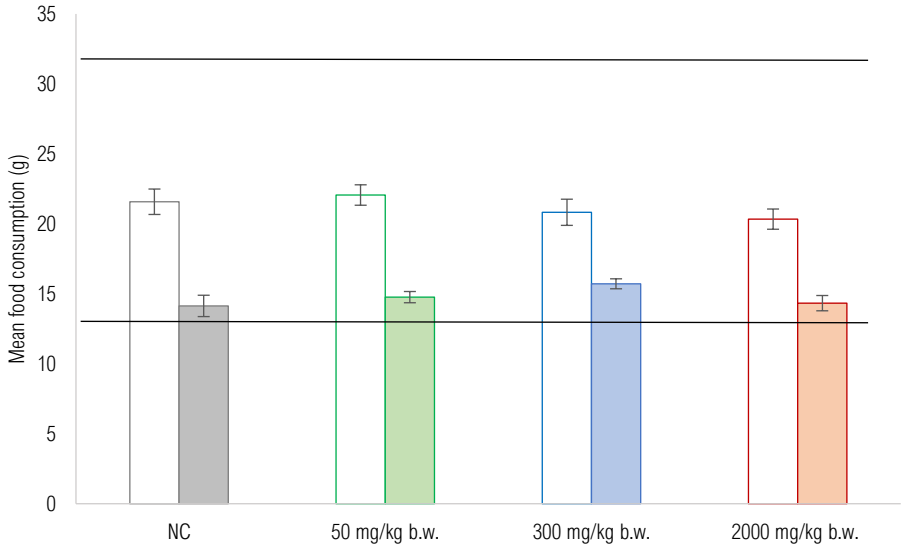

Figure 16. Results of the repeated dose 28-day oral toxicity study food consumption. The Figure shows the mean food consumption ± SD of each group during the whole study. Data are represented in empty columns for males and in filled columns for females. The normal food consumption limits established by the animal supplier are represented by the black lines. NC: negative control.

The hematological parameters showed statistically significant differences in several parameters in comparison with their correspondent negative control values (Table 16). Statistically significant differences were found in some random parameters of the differential and absolute white blood cell count (Table 17), biochemical analysis (Table 18), and coagulation (Table 19). The urinalysis performed did not show relevant alterations in the evaluation of glucose, bilirubin, ketone bodies, density, erythrocytes, pH, proteins, urobilinogen, nitrites, and

leukocytes (Tables 20 and 21). Furthermore, volume, appearance, colour, and odour of urine were found to be normal (data not shown).

Table 16. Results of the hematological evaluation of the repeated dose 28-day oral toxicity study. The table collects the group mean ± SD for each parameter evaluated. According to the levels of significance: (*) significant ($p < 0.05$).

	RBC ($x10^6$ cel/ml)	WBC ($x10^3$ cel/ml)	Hb (g/dl)	Htc (%)	MCV (fl)	MCH (pg)	MCHC (g/dl)	Platelets ($x10^3$ cel/ml)	Ret (%)
Females									
NC	8.24 ± 0.33	5.32 ± 1.24	15.0 ± 0.6	42.8 ± 1.9	51.9 ± 0.7	18.2 ± 0.1	35.0 ± 0.5	803 ± 98	2 ± 1
50 mg/kg	8.05 ± 0.27	3.66 ± 0.92	14.9 ± 0.3	42.1 ± 0.	52.3 ± 1.3	18.5 ± 0.5	35.3 ± 1.0	652 ± 159	1 ± 1
300 mg/kg	8.12 ± 0.20	2.72 ± 1.18*	14.9 ± 0.4	42.3 ± 1.1	52.1 ± 0.5	18.3 ± 0.5	35.2 ± 0.6	733 ± 129	1 ± 0
2000 mg/kg	8.22 ± 0.50	3.71 ± 0.60	15.1 ± 0.7	43.1 ± 2.3	52.5 ± 1.3	18.4 ± 0.8	35.1 ± 1.4	655 ± 135	1 ± 1
Males									
NC	9.44 ± 0.44	5.66 ± 0.94	16.1 ± 0.3	44.6 ± 0.5	48.7 ± 1.4	17.5 ± 0.7	36.1 ± 0.7	712± 59	1 ± 1
50 mg/kg	8.87 ± 0.46	6.24 ± 1.06	16.3 ± 0.7	45.4 ± 2.4	49.5 ± 0.4	17.8 ± 0.2	35.9 ± 0.4	832 ± 72	2 ± 1
300 mg/kg	9.19 ± 0.51	6.68 ± 0.21	16.4 ± 0.6	44.9 ± 1.6	50.6 ± 1.8	18.5 ± 0.6*	36.6 ± 0.2	830 ± 118	1 ± 1
2000 mg/kg	9.17 ± 0.35	6.60 ± 1.09	16.7 ± 0.4	47.2 ± 1.6	50.1 ± 1.4	17.7 ± 0.5	35.4 ± 0.8	683 ± 193	1 ± 1

NC: negative control, (): measurement unit. RBC: red blood cell count, WBC: white blood cell count, Hb: hemoglobin, Htc: hematocrit, MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, CHMC: corpuscular hemoglobin mean concentration.

Table 17. Results of the absolute and differential count of the repeated dose 28-day oral toxicity study. The table collects the group mean ± SD for each parameter evaluated. According to the levels of significance: (*) significant ($p < 0.05$).

	Eosinophils		Neutrophils		Lymphocytes		Monocytes		Basophils	
	$x10^3$ cell/mL	%	$x10^3$ cell/mL	%	$x10^3$ cell/mL	%	$x10^3$ cell/mL	%	$x10^3$ cell/mL	%
Females										
NC	0.06 ± 0.02	1.4 ± 0.7	0.58 ± 0.24	12.4 ± 4.6	4.00 ± 1.33	82.1 ± 6.3	0.21 ± 0.17	4.1 ± 2.8	0.00 ± 0.00	0.0 ± 0.0
50 mg/kg	0.04 ± 0.01*	1.2 ± 0.4	0.83 ± 0.06	25.4 ± 2.9	2.31 ± 0.48*	69.6 ± 3.4	0.13 ± 0.04	3.9 ± 1.1	0.00 ± 0.00	0.0 ± 0.0
300 mg/kg	0.04 ± 0.01	2.0 ± 1.0	0.49 ± 0.10	22.3 ± 14.2	2.07 ± 1.12*	71.5 ± 16.0	0.11 ± 0.04	4.3 ± 1.4	0.00 ± 0.00	0.0 ± 0.0
2000 mg/kg	0.07 ± 0.02	1.7 ± 0.4	0.64 ± 0.12	16.1 ± 2.8	3.07 ± 0.31	78.0 ± 3.5	0.17 ± 0.02	4.2 ± 0.8	0.00 ± 0.00	0.0 ± 0.0
Males										
NC	0.12 ± 0.11	1.9 ± 1.8	1.19 ± 0.42	19.5 ± 5.7	4.60 ± 1.17	73.6 ± 6.4	0.30 ± 0.12	5.0 ± 1.9	0.00 ± 0.00	0.0 ± 0.0
50 mg/kg	0.08 ± 0.05	1.2 ± 0.6	1.42 ± 0.64	20.9 ± 8.2	4.78 ± 0.99	71.8 ± 9.9	0.40 ± 0.12	6.0 ± 1.5	0.00 ± 0.00	0.0 ± 0.0
300 mg/kg	0.06 ± 0.02	1.0 ± 0.5	0.86 ± 0.12	14.2 ± 3.1	5.01 ± 1.05	79.8 ± 3.3	0.31 ± 0.06	3.9 ± 2.1	0.00 ± 0.00	0.0 ± 0.0
2000 mg/kg	0.09 ± 0.06	1.5 ± 0.9	0.96 ± 0.32	16.6 ± 5.5	4.62 ± 0.99	77.8 ± 7.0	0.23 ± 0.16	4.3 ± 3.4	0.00 ± 0.00	0.0 ± 0.0

NC: negative control; (): measurement unit.

Table 18. Results of the biochemical analysis of the repeated dose 28-day oral toxicity study. The table collects the group mean ± SD for each parameter evaluated. According to the levels of significance: (*) significant ($p < 0.05$) and (**) very significant ($p < 0.01$).

	NC	50 mg/kg	300 mg/kg	2000 mg/kg	NC	50 mg/kg	300 mg/kg	2000 mg/kg
	Females				Males			
ALB (g/dL)	4.7±0.3	4.3 ±0.1*	4.8±0.3	4.8±0.4	4.3±0.2	4.7±0.4	4.2±0.2	4.3 ±0.2
Urea (mg/dL)	40±7	37±2	37±4	38±4	33±4	38±4	34±12	30±2
AST (U/L)	84±17	93±8	72±10	91±24	77±10	82±6	89 ±4*	92 ±3**
ALT (U/L)	21±4	22±5	18±3	18±5	23±2	18 ±2**	22±2	22±4
ALP (U/L)	48±8	92±17**	58±14	47±9	108±15	47 ±6**	106±24	104±17
BIL-T (mg/dL)	0.13±0.02	0.11±0.01	0.10±0.02	0.11±0.02	0.11±0.03	0.09±0.02	0.10±0.02	0.11±0.03
CHOL (mg/dL)	69±4	81 ±11*	75±16	68±18	77±11	74±16	70±5	78±7
GLU (mg/dL)	95±12	107±17	103±17	84±10	107±11	111±22	115±22	102±16
CREA (mg/dL)	0.43±0.05	0.40±0.08	0.43±0.05	0.43±0.05	0.34±0.02	0.40±0.08	0.35±0.05	0.34±0.05
TP (g/dL)	6.4±0.3	6.2±0.2	6.3±0.4	6.5±0.2	6.2±0.3	6.2±03	6.0±0.2	6.0±0.3
CPK (U/L)	504±147	770 ±153*	407±84	633±293	546±219	525±76	756±100	726±102
Ca (mg/dL)	9.72±0.43	10.38±0.42	10.39±0.56	10.24±0.12	9.94±0.31	10.20±0.25	10.19±0.18	10.15±0.16
TG (mg/dL)	36±11	54 ±13*	35±10	37±7	62±43	39±3	57±10	56±7
Cl (mg/dL)	95.6±0.8	101.86 ±1.36**	104.72 ±1.63**	103.94 ±2.61**	93.86±1.24	101.9 ±1.0**	102. 3 ±0.6**	105.5 ±5.4**
K (mg/dL)	4.02±0.29	3.91±0.13	4.19±0.28	4.28±0.21	4.28±0.20	4.50±0.31	4.56±0.31	4.94 ±0.24**
Na (mg/dL)	133±3	142 ±1**	135±3	138±4	132±9	145 ±2*	140±8	145 ±8*
Glob (g/dL)	1.7±0.2	1.9±0.1	1.6±0.2	1.7±0.2	2.0±0.2	1.7 ±0.1*	1.8±0.2	1.7 ±0.1*

NC: negative control; (): measurement unit. ALB: Albumin. AST: aspartate transaminase. ALT: alanine transaminase. ALP: alkaline phosphatase. BIL-T total bilirubin. CHOL: total cholesterol. GLU: glucose. CREA: creatinine. TP: total protein. CPK: creatine phosphokinase. Ca: calcium. TG: triglycerides. Cl: chlorine. K: potassium. Na: sodium. Glob: globulin.

Table 19. Results from the coagulation analysis of the repeated dose 28-day oral toxicity study. The table collects the group mean ± SD for each parameter evaluated. According to the levels of significance: (*) significant (p <0.05) and (**) very significant (p < 0.01).

Group	Fibrinogen (mg/dL)	PT (sg)	aPTT (sg)	Fibrinogen (mg/dL)	PT (sg)	aPTT (sg)
	Females			Males		
NC	149.2 ± 9.5	16.4 ± 1.1	29.8 ± 5.3	209.6 ± 9.8	17.0 ± 0.8	29.0 ± 4.9
50 mg/kg	179.2 ± 23.2*	13.9 ± 0.3	31.1 ± 3.5	215.7 ± 10.6	14.9 ± 0.3**	28.6 ± 1.3
300 mg/kg	186.0 ± 12.2**	14.4 ± 0.6	24.7 ± 3.4	233.6 ± 17.7	16.3 ± 3.0	30.4 ± 13.0
2000 mg/kg	139.5 ± 39.4	12.8 ± 1.8	24.4 ± 9.3	220.3 ± 19.4	14.8 ± 1.1*	24.6 ± 3.3

NC: negative control; (): measurement unit. PT prothrombin time. aPTT: activated partial thromboplastin time.

Table 20. Results of the urinary biochemistry of the females of the repeated-dose 28-day oral toxicity study. The table collects for each animal the individual data obtained in the urinalysis. •: values outside the laboratory records. Normality ranges (bold letter) were calculated from the laboratory histories (n = 49 animals).

Group	Animal	Density (mg/dL) **1.001-1.023**	pH **5.8-8.2**	Leucocytes (cel/µL)	Nitrites	Proteins (mg/dL)	Glucose (mg/dL)	Ketonic bodies (mg/dL)	Urobilinogen (mg/dL)	Bilirubin (mg/dL)	Erythrocytes (cel/µL)
NC	F1	1.010	7	Neg	Pos	Neg	Nor	Neg	Nor	Neg	Neg
	F2	1.015	6.5	Neg	Pos	Neg	Nor	Neg	Nor	Neg	Neg
	F3	1.025	5•	25/µL	Neg	Pos	Nor	15 mg/dL	Nor	1 mg/dL	10 /µL
	F4	1.010	7	Neg	Pos	Neg	Nor	Neg	Nor	Neg	Neg
	F5	1.015	6	Neg	Pos	Neg	Nor	Neg	Nor	Neg	Neg
50mg/kg b.w.	F1	1.015	6.5	Neg	Neg	Neg	Nor	Neg	Nor	Neg	Neg
	F2	1.020	6	Neg	Neg	25 mg/dL	Nor	5 mg/dL	Nor	Neg	Neg
	F3	1.005	7	Neg	Neg	Neg	Nor	Neg	Nor	Neg	Neg
	F4	1.015	7	Neg	Neg	Neg	Nor	Neg	Nor	Neg	Neg
	F5	1.015	7	25 /µL	Neg	25 mg/dL	Nor	5 mg/dL	Nor	Neg	Neg
300 mg/kg b.w.	F1	1.010	6.5	Neg	Neg	Neg	Nor	Neg	Nor	Neg	Neg
	F2	1.010	7	Neg	Neg	Neg	Nor	Neg	Nor	Neg	Neg
	F3	1.005	7	Neg	Neg	Neg	Nor	Neg	Nor	Neg	Neg
	F4	1.015	7	Neg	Neg	Neg	Nor	Neg	Nor	Neg	Neg
	F5	1.010	7	Neg	Neg	Neg	Nor	Neg	Nor	Neg	Neg
2000 mg/kg b.w.	F1	1.020	6.5	Neg	Neg	25 mg/dL	Nor	Neg	Nor	Neg	Neg
	F2	1.020	6.5	Neg	Neg	25 mg/dL	Nor	Neg	Nor	Neg	Neg
	F3	1.025	5•	Neg	Neg	25 mg/dL	Nor	5 mg/dL	Nor	Neg	Neg
	F4	1.015	6.5	Neg	Neg	25 mg/dL	Nor	Neg	Nor	Neg	Neg
	F5	1.015	6.5	Neg	Neg	Neg	Nor	5 mg/dL	Nor	Neg	Neg

NC: negative control; Pos: positive. Neg: negative. Nor: Normal.

Table 21. Results of the urinary biochemistry of the males of the repeated-dose 28-day oral toxicity study. The table collects for each animal the individual data obtained in the urinalysis. Normality ranges (bold letter) were calculated from the laboratory histories (n = 65 animals).

Group	Animal	Density (mg/dL) **1.001-1.023**	pH **5.8-8.2**	Leucocytes (cel/µL)	Nitrites	Proteins (mg/dL)	Glucose (mg/dL)	Ketonic bodies (mg/dL)	Urobilinogen (mg/dL)	Bilirubin (mg/dL)	Erythrocytes (cel/µL)
NC	M1	1.010	8	25/µL	Neg	25 mg/dL	Nor	Neg	Nor	Neg	Neg
	M2	1.010	7	25/µL	Pos	25 mg/dL	Nor	5 mg/dL	Nor	Neg	Neg
	M3	1.015	7	25/µL	Neg	25 mg/dL	Nor	15mg/dL	Nor	Neg	10/µL
	M4	1.005	7	Neg	Neg	Neg	Nor	Neg	Nor	Neg	Neg
	M5	1.005	7	Neg	Neg	Neg	Nor	Neg	Nor	Neg	Neg
50mg/kg b.w.	M1	1.010	8	25/µL	Neg	25 mg/dL	Nor	Neg	Nor	Neg	10/µL
	M2	1.015	6.5	100/µl	Neg	75 mg/dL	Nor	15 mg/dL	Nor	1 mg/dL	25/µL
	M3	1.010	7	Neg	Neg	25 mg/dL	Nor	Neg	Nor	Neg	Neg
	M4	1.005	7	Neg	Neg	25 mg/dL	Nor	Neg	Nor	Neg	Neg
	M5	1.005	7	25/µL	Neg	25 mg/dL	Nor	Neg	Nor	Neg	10/µL
300 mg/kg b.w.	M1	1.005	7	25/µL	Neg	25 mg/dL	Nor	Neg	Nor	Neg	10/µL
	M2	1.015	7	100/µL	Neg	75 mg/dL	Nor	15 mg/dL	Nor	1 mg/dL	10/µL
	M3	1.010	7	Neg	Neg	25 mg/dL	Nor	Neg	Nor	Neg	Neg
	M4	1.005	7	Neg	Neg	25 mg/dL	Nor	Neg	Nor	Neg	Neg
	M5	1.010	8	Neg	Neg	25 mg/dL	Nor	Neg	Nor	Neg	Neg
2000 mg/kg b.w.	M1	1.010	8	25/µL	Pos	25 mg/dL	Nor	Neg	Nor	Neg	10/µL
	M2	1.005	7	25/µL	Neg	25 mg/dL	Nor	15 mg/Ll	Nor	Neg	Neg
	M3	1.005	7	25/µL	Neg	25 mg/dL	Nor	Neg	Nor	Neg	Neg
	M4	1.005	7	Neg	Pos	Neg	Nor	Neg	Nor	Neg	Neg
	M5	1.010	8	Neg	Neg	Neg	Nor	Neg	Nor	Neg	Neg

NC: negative control; Pos: positive. Neg: negative. Nor: Normal.

The external examination of the abdominal and thoracic cavities and the extracted target organs did not show relevant macroscopic alterations. Besides, in the pathological study, no microscopic alterations were found in the extracted target organs. Statistically significant differences in the absolute or relative weight of the organs were not detected (data not shown). All the findings were within the normal background for 12–14-week-old Wistar rats. Due to the absence of toxic effects in histopathological examinations of the negative control and 2000 mg/kg b.w. groups of the principal study, the examinations of the 50 mg/kg b.w. and 300 mg/kg b.w. groups were not carried out.

Considering that no toxic effects were found in the principal study groups, the results of the reversion study were analysed but are not shown.

3.3. Micronucleus test

The induction of chromosomal aberrations was assessed by the MN test, in bone marrow samples, obtained from the female rats of the principal groups of the 28-day oral toxicity study, following the principles of the OECD TG 474 (OECD, 1997b). The ratio of PCE/NCE was determined by counting 500 PCE+NCE. The total MN were determined by counting at least 4000 PCE; then the MN % was determined. The raw data are presented in Table 22 and the ratio in figure 17.

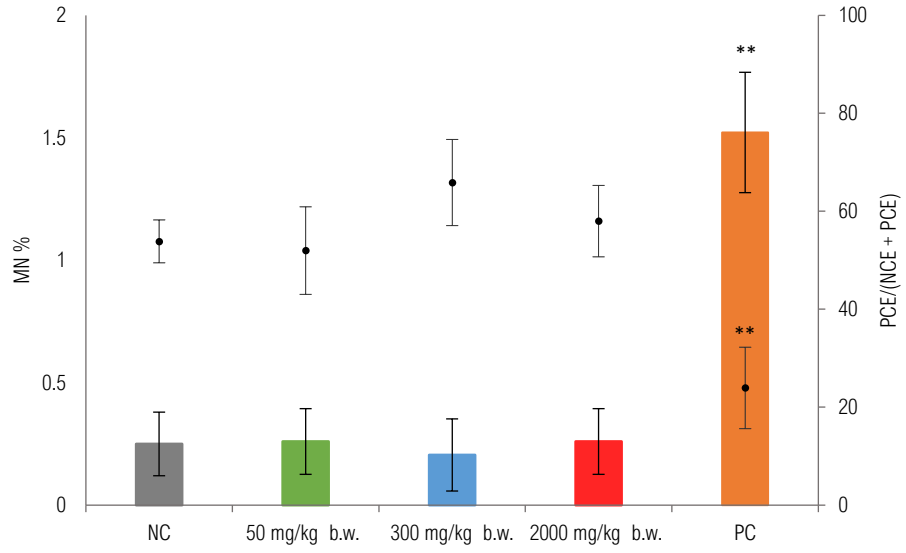

Figure 17. Results of the *in vivo* erythrocyte MN test. The graphic shows the mean ± SD of the MN % in bars and the PCE over the EPC+NCE (dots) for each group. **: (P< 0.01) statistically significant different from negative control. NC: negative control, PC: positive control.

Mitomycin C at a dose of 4 mg/kg promoted a statistically significant induction of MN in comparison to the negative control, showing mean values of 1.5 ± 0.2% and 0.3 ± 0.1%, respectively. Neither silver-kaolin formulation treatment group showed a significant decrease in the PCE/ (NCE +PCE) or a statistically significant increase in MN %.

Table 22. Raw data of the results of the *in vivo* erythrocyte MN test.

Group ID	PCE	NCE*	PCE/ (NCE +PCE)	PCE	MN	MN %
	238	262	47.6	4000	10	0.25
	263	237	52.6	4000	3	0.075
	303	197	60.6	4000	7	0.175
	272	228	54.4	4000	15	0.375
NC	261	239	52.2	4000	15	0.375
	288	212	57.6	4000	14	0.35
	237	263	47.4	4000	3	0.075
	218	282	43.6	4000	17	0.425
	322	178	64.4	4000	9	0.225
50 mg/kg b.w.	227	273	45.4	4000	9	0.225
	301	199	60.2	4000	6	0.15
	305	195	61	4000	15	0.375
	313	187	62.6	4000	3	0.075
	407	93	81.4	4000	14	0.35
300 mg/kg b.w.	319	181	63.8	4000	3	0.075
	291	209	58.2	4000	17	0.425
	312	188	62.4	4000	13	0.325
	301	199	60.2	4000	15	0.375
	227	273	45.4	4000	5	0.125
2000 mg/kg b.w.	316	184	63.2	4000	13	0.325
	85	415	17	4139	74	1.788
	71	429	14.2	4000	65	1.625
	173	327	34.6	4000	49	1.225
	134	366	26.8	4000	5	0.125
PC	147	353	29.4	4001	52	1.300

NC: negative control; PC: positive control; PCE: polychromic erythrocytes; NCE: normochromic erythrocytes; *Number of NCE obtained after counting 500 (PCE+NCE); MN: micronuclei.

3.4. Standard comet assay

The presence of SBs or ALS was assessed by the standard comet assay in fresh liver, kidney, spleen and duodenum samples, of the female rats of the principal groups of the 28-day oral toxicity study, following the principles of the OECD TG 489 (OECD, 2016a). Results are shown in Figure 18.

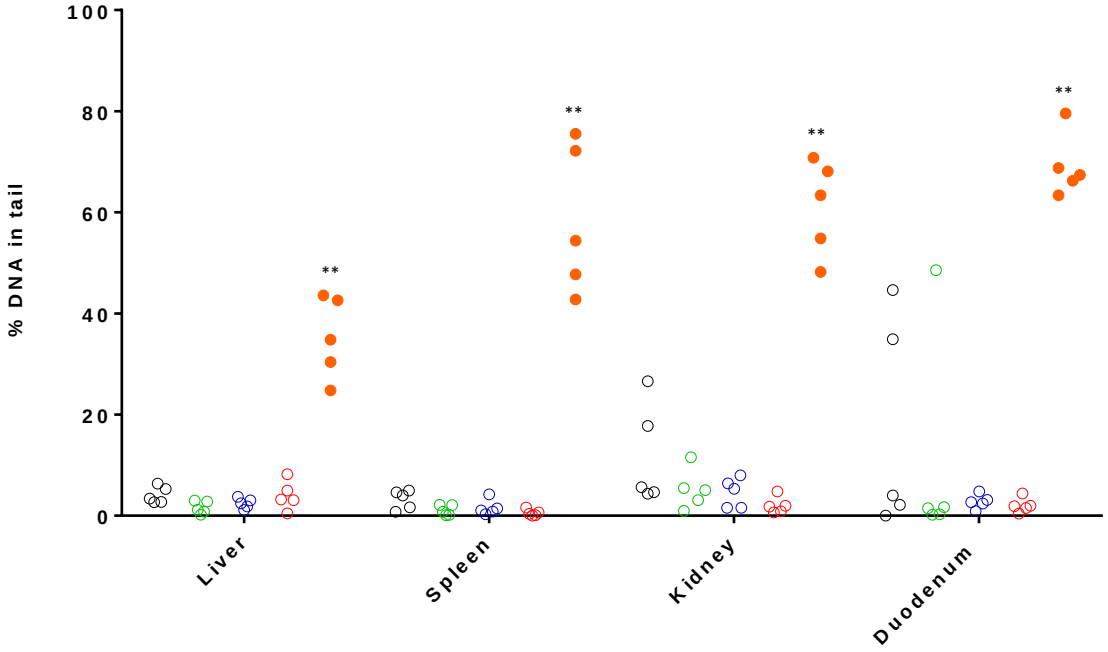

Figure 18. Results of the *in vivo* standard comet assay in fresh samples of liver, spleen, kidney and duodenum. The graphic shows the % DNA in tail of each five animals composing negative control (grey), 50 mg/kg b.w. (green), 300 mg/kg b.w. (blue), 2000 mg/kg b.w. (red) and positive control (full orange). **: (P< 0.01) statistically significantly different from negative control.

MMS, used as a positive control, at a dose of 200 mg/kg b.w. promoted a statistically significant induction of DNA damage than that induced by the vehicle (distilled water), accounting for mean values of 35.23 ± 8.02% in liver, 58.53 ± 14.65% in spleen, 61.08 ± 9.41% in kidney and 69.06 ± 6.18% in duodenum. Negative controls showed values of 4.09 ± 1.66% in liver, 3.21 ± 1.87% in spleen, 11.80 ± 9.98% in kidney and 17.13 ± 20.99% in duodenum. Regarding the results of the silver-kaolin formulation treatments, none of the organs after any of the treatments showed a statistically significant increase in % DNA in tail compared with the negative control. Highly damaged comets were found only in few samples and the values were very low, therefore there were considered as not relevant (data not shown).

3.5. Fpg-modified comet assay

The induction of Fpg-sensitive sites (i.e., oxidized bases) was assessed by the Fpg-modified comet assay in frozen liver, kidney, spleen and duodenum samples of negative control and high dose groups. Results are shown in Figure 19. In the high dose group, only three % DNA in tail data were obtained to be evaluated in liver, since two of the samples did not contain enough cells to be scored.

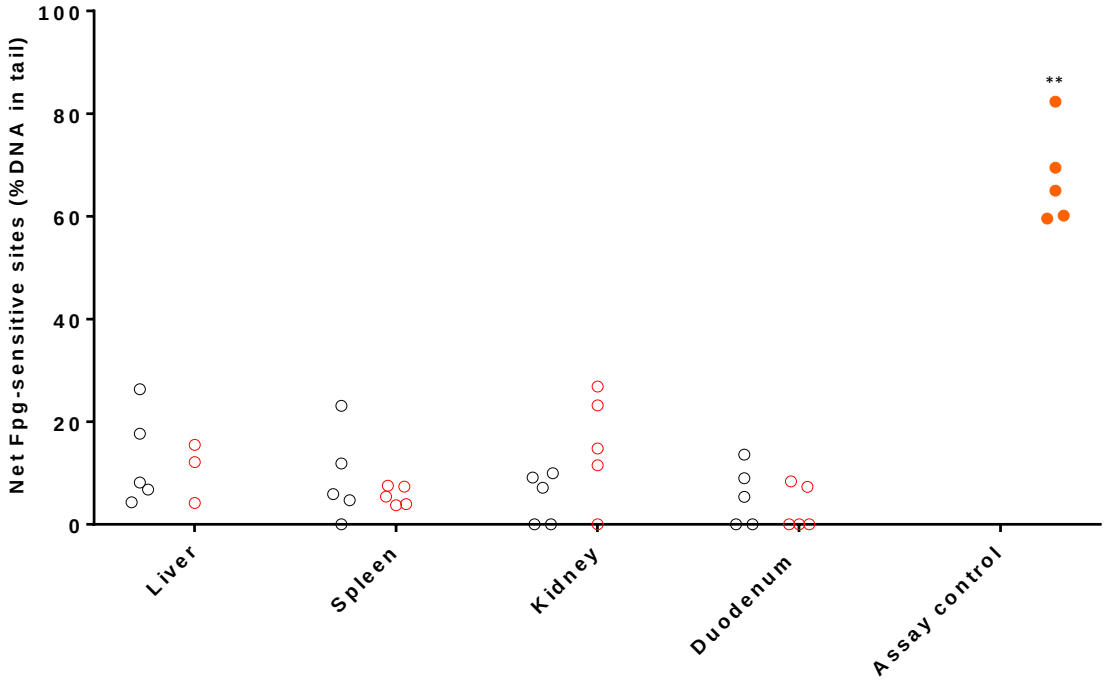

Figure 19. Results of the *in vivo* Fpg-modified comet assay in frozen samples of liver, spleen, kidney, and duodenum. The graphic shows the Fpg-sensitive sites in terms of % DNA in tail of each five animals composing negative control (grey), 2000 mg/kg b.w. (red) and assay control (full orange). **: (P< 0.01) statistically significant different from negative control.

TK6 cells treated with 1.25 mM $KBrO_3$ for 3 h, used as positive control, promoted a net Fpg-sensitive sites according to expectations, accounting for a mean value of 67.30 ± 9.30% DNA in tail. Besides, no significant differences in the induction of Fpg-sensitive sites were found for each tissue in comparison with their respective negative control.

Chapter 5. Discussion

The silver-kaolin formulation evaluated in the present PhD thesis was designed and prepared to be used as a feed additive based on the known bactericidal effect of silver. This formulation could be a safer alternative developed in response to the growing problem posed by the worldwide AMR. Silver kaolin formulation was developed by ENOSAN (Spain); commercial kaolin is treated through a method under patent, enabling silver (Ag) to be deposited on its surface as silver nanoparticles (AgNPs). The resulting material is based in bactericidal AgNPs embedded in the surface of a kaolin matrix (Feed additive for animals: Spanish National patent Nº 200701496 and Nanosystems comprising silver and antibiotics and their use for the treatment of bacterial infections: Patent in international extension China, Japan, USA, EPO, Brazil, Colombia and Mexico, Nº PCT/EP2018/059006).

The physicochemical characterization of the silver-kaolin formulation was carried out by the "Group of Analytical Spectroscopy and Sensors" of the University of Zaragoza. The formulation was composed by a laminar structure of kaolinite microparticles decorated with spheroidal AgNPs with diameters ranging from 2 to 90 nm (average diameter 27 nm). Silver-kaolin formulation consisted in a crystal phase composed of 68% kaolinite, 12% quartz, 13% illite, 6% potassic feldspar and 0.8% metallic silver.

The "Microbiology and Parasitology Department" of the University of Navarra studied the *in vitro* antibacterial activity of the silver-kaolin formulation (Pérez-Etayo et al., 2021). They confirmed the antimicrobial activity of the formulation against a wide selection of gram-positive and gram-negative bacteria, including resistant and multi-resistant ones, being more effective against gram-negative bacteria. Furthermore, they evidenced the lack of activity of the kaolin formulation without AgNPs.

AgNPs-kaolin based materials have been developed in the last years, also demonstrating their effectiveness as antimicrobials in *in vitro* studies (Awad et al., 2021; Hariram et al., 2021; Hassouna et al., 2017; Ojer-Usoz et al., 2014). Furthermore, the *in vivo* antimicrobial efficacy of these materials suggests that these materials could be used as dietary supplements in animal feed (Dosoky et al., 2021; Fondevila et al., 2009; Latorre and Fondevila, 2010; Su et al., 2012). The immobilization of AgNPs on inert matrixes as kaolin or other clays to obtain silver-clay based materials seems to enhance their antimicrobial activity.

Once the efficacy has been evaluated, it is also necessary to evaluate the safety of the material. This thesis is mainly focused on the genotoxicity evaluation of the material. First, a bibliographic revision of the information available regarding the genotoxicity of AgNPs has been carried out. Then, as an essential part of silver-kaolin formulation safety assessment, *in vitro* and *in vivo* genotoxicity studies have been carried out, following the corresponding EFSA guideline testing strategy (EFSA Scientific Comittee et al., 2018). Moreover, by integrating the *in vivo* genotoxicity studies in a repeated dose toxicity study, the *in vivo* toxicity of the material was also

evaluated. Each assay was conducted following the principles of their corresponding OECD guidelines and under GLPs-like conditions.

1. Bibliographic revision of the genotoxicity of silver nanoparticles

When the results of the *in vivo* and *in vitro* assays are considered as a whole, it is evident that positive results were obtained for every damage level (i.e., primary DNA damage, gene mutations and chromosomal aberrations), although it is important to note that positive results were predominant in the *in vitro* assays and negative results in the *in vivo* assays. The few exceptions to this rule were obtained with cell lines that are not normally used in the *in vitro* MN assay (HBEC, MCF-10A, MCF-7, MDA-MB-231 and BALB/3T3A31-1-1). The *in vivo* results as a whole show that MN and chromosomal aberration assays obtained more positive results than the comet assays. Thus, AgNPs also had the capacity to induce chromosomal aberrations *in vivo*. By contrast, DNA damage was not generally detected in bone marrow, liver, lung or testis with the standard comet assay, but the enzyme-modified versions, which detect mainly oxidized bases, produced some positive results. Moreover, a number of factors such as AgNPs biodistribution (see below) and the experimental *in vivo* design may account for these results.

None of the articles selected carried out a comprehensive evaluation of AgNPs genotoxicity through a complete battery of assays that included both *in vitro* and *in vivo* experimental designs, as recommended in the EFSA strategy. Only Wang et al. (2019) applied a strategy that included both *in vitro* and *in vivo* assays; they evaluated AgNPs genotoxicity through the *in vitro* MN and comet tests and the *in vivo* bone narrow MN test, and obtained positive results in all of them. Guo et al. (2016) studied the genotoxicity of AgNPs by applying a core battery of *in vitro* genotoxicity assays recommended for the Food and Drug Administration regulated products: the Ames test, the MLA and the *in vitro* MN assay. They obtained positive results in all except the Ames test, which produced inconclusive results, thereby confirming that this assay is not suitable for NPs in general, especially for AgNPs.

Biodistribution analysis is essential to understand the behavior of NPs in organisms and to select the tissue to be analyzed for genotoxicity. In general, AgNPs accumulate primarily in the mononuclear phagocyte system such as liver and spleen after both oral (Van der Zande et al., 2012) and intravenous administration (Yang et al., 2017). Van der Zande et al. 2012 showed that after 28 days of oral administration of AgNPs, low concentrations of AgNPs passed through the intestines and AgNPs were eliminated from most organs, except from the brain and testis, eight weeks after treatment. AgNPs biodistribution was not studied in all *in vivo* genotoxicity assays retrieved in this review, but when the test was performed, the results were consistent even if the dose, administration route and treatment schedule differed. Silver has been detected in bone marrow,

blood and liver (Li et al., 2014; Martins et al., 2017; Wen et al., 2017), which are the most commonly used tissues in genotoxicity assays.

AgNPs attach to the cell membrane where they alter permeability and penetrate the cell, thus releasing Ag+ ions (Burdușel et al., 2018; Dakal et al., 2016). Once inside the cell, Ag+ ions cause damage to structures and biomolecules, alterations in respiration, oxidative stress by ROS and modulation of signal transduction pathways. One of the mechanisms through which NPs cause cell damage, is based on the exogenous generation of ROS such as superoxide anion, hydroxyl radical, singlet oxygen, hypochlorous acid and hydrogen peroxide which can damage DNA and lead to mutagenesis processes (Abdal Dayem et al., 2017; AshaRani et al., 2009; Che et al., 2017; Magdolenova et al., 2014; McShan et al., 2014). AgNPs can also interact with mitochondria and disrupt the electron transport chain, thereby causing ROS production and interrupting of ATP synthesis; ROS increase and a shortage of ATP result in protein and DNA damage (Asharani et al., 2009; McShan et al., 2014).

Ag^+ ions released from the AgNPs are bioactive; they have a specific capacity to form complexes with proteins by thiol groups (Nallanthighal et al., 2017a; Salim et al., 2019). Ag^+ ions in complexes with proteins can interfere with Na^+/Cl^- transport and contribute to ROS production, disrupt the physiological activity of proteins and even lead to cell death (Burdușel et al., 2018; Nallanthighal et al., 2017a; Salim et al., 2019). Whether the effects observed in AgNPs testing are produced by the NPs themselves or by the ions that are released is not clear and requires further study (Nallanthighal et al., 2017a; Salim et al., 2019).

In addition, AgNPs have been shown to damage cell membranes disrupt the DNA repair cell systems, and cause the depletion of antioxidant molecules (McShan et al., 2014). Furthermore, interaction between AgNPs and DNA can cause DNA shearing or denaturation and disrupt cell division (Dakal et al., 2016).

The results of both *in vitro* and *in vivo* comet assays may indicate that the genotoxic effect is produced by oxidative mechanism, but it is difficult to conclude something since the studies that evaluated this damage were scarce. According to Kain et al. (2012), the interactions of NPs with the SH groups at the active site of Fpg, can reduce the enzyme capacity to detect damage. On the other hand, Magdolenova et al. (2014) claimed that in the actual comet assay, Fpg do not interact directly with NPs, therefore they do not affect Fpg activity. The positive Fpg comet results obtained with AgNPs confirm this (Table 5). It is worth noting that only a few papers contained results obtained with the enzyme-modified comet assay, four of them *in vitro* (Ávalos et al., 2015; Franchi et al., 2015; Roszak et al., 2017; Vila et al., 2018) and three of them *in vivo* (Asare et al., 2016; Li et al., 2014; Patlolla et al., 2015). More studies are required to shed light on the genotoxic mechanism of action of AgNPs however, three of the four *in vitro* assays and two of the three *in vivo* assays reported positive results.

An analysis of the tables suggests that AgNPs size does not influence the genotoxicity of AgNPs, however, this may be due to the way in which doses are expressed. When doses are measured in mg/mL or mg/kg, the surface area of the particles is not considered. AgNPs activity is better correlated with surface area concentration than with mass concentration (Sotiriou et al., 2011). Guo and colleagues (2016) observed that when results are corrected by surface area, the differences decrease; if the particles are larger, the same dose contains fewer of them and therefore, has a lower surface area and activity. Mass concentration expressed in mg/mL can be converted to surface area concentration expressed in mm^2/mL using the size and particle number (Guo et al., 2016).

Some authors claim that the genotoxic effect of AgNPs is influenced by their coating (Guo et al., 2016; Nallanthighal et al., 2017a). Citrate-coated AgNPs have an increased negative charge which results in high stability and decreases aggregation (Rageh et al., 2018). Theoretically, the small size and active surface of citrate-coated AgNPs allow them to penetrate the mitochondria and nuclear pore and to create free radicals (Patlolla et al., 2015). With regard to *in vivo* MN test, silicon coating produced negative results in Li et al. (2014) and PVP-coating produced negative results in almost every evaluation (Li et al., 2014; Nallanthighal et al., 2017a) except for Wang et al. (2019). Moreover, Nallanthighal et al. (2017a) obtained positive MN results after seven days of oral treatment with citrate coated AgNPs in C57BL/6 mice, but negative results after treatment with PVP-AgNPs under the same conditions. Regarding the *in vivo* comet assay, silicon- and PVP- coatings produced negative results in the standard comet assay, although Li et al. (2014) obtained positive results in Endo-III and OGG-1 modified comet assays.

A physicochemical characterization of NMs is essential to understand their behavior. In the analyzed papers, the main parameters determined were particle size and size diameter, hydrodynamic diameter, polydispersity index, zeta potential, particle morphology and agglomeration state. Dynamic light scattering was one of the most widely used techniques to determine particle size, size distribution, zeta potential, hydrodynamic diameter and polydispersity index. Transmission Electronic Microscopy was used to determine particle size, morphology, particle shape and agglomeration. Zeta potential was determined mostly with a Zetasizer instrument. Other techniques such as Inductively Coupled Plasma Mass Spectrometry and X-ray Photoelectron Spectroscopy were applied to determine AgNPs concentrations and elemental composition, respectively.

Looking at all the results together, it appears to indicate that the AgNPs can produce genotoxic effects. Positive results were obtained in all type of assays, both *in vitro* and *in vivo*, although characteristics of each AgNPs and test conditions should be considered case-by-case. AgNPs, as previously mentioned in the introduction, are highly used in consumer products, medicine and other sectors and this poses a worrying scenery that needs complete risk assessment evaluating real human exposure.

2. *In vitro* evaluation

2.1. Methods adaptation; washing procedures

NMs are known to cause interferences with the *in vitro* assays (Drasler et al., 2017; Kroll et al., 2009). But interferences are not the only problem to face when testing NMs. There is a critical point during *in vitro* assays to test AgNPs, especially when cells in suspension are used. After treatment, cells are generally centrifuged to remove the AgNPs, but in many cases, complete separation of solid AgNPs from the cells may not be possible. This is an important factor that can affect results, especially in assays in which the results are not evaluated immediately after treatment but following an incubation period. In the MLA, for example, the treatment period (3–4 h or 24 h), is followed by the subculturing for 48h and cell seeding for colony growth for 12–14 days; if the NPs have not been completely removed, the treatment period can be extended from 3–4 h or 24h to as much as 17 days. This is a technical factor that may increase *in vitro* sensitivity.

Removing the NMs from the *in vitro* models can also be a challenge. Silver-kaolin formulation is a poorly soluble material and the usual centrifugation conditions to wash the cells after treatment were not useful, as the material was isolated along with the cells. Several methods to separate L5178Y $TK^{+/-}$ cells from the silver-kaolin formulation were tried: calculations for differential centrifugation, Lymphoprep™, filtration and decantation. Five minutes of decantation allowed the recovery of 100% of the L5178Y $TK^{+/-}$ cells and the elimination of the maximum amount of silver-kaolin formulation (Figure 9). However, this method was suitable when using a relatively high volume of cell culture for the cell treatment, as it is the case of the MLA, but not for small volumes, as it is the case of MN and the comet assays. In these assays, TK6 cells were centrifuged after treatment, despite not removing the silver-kaolin formulation completely. This implies that part of the silver-kaolin formulation was present in the post-treatment incubation period of the cytotoxicity and proliferation assays, and in the MLA, although after their corresponding sub-culture steps, the amount of formulation remaining was decreased. For this reason, silver-kaolin formulation was not evaluated through the MN test after 3 h treatment; if the product is not eliminated after centrifugation, the 3 h treatment would become a 24 h treatment. Therefore, decantation was the method used in the assays performed in L5178Y $TK^{+/-}$ (MLA) and centrifugation in the ones performed in TK6 cells (MN and comet assays).

Furthermore, considering that AgNPs are the active compound of the formulation, its release, after 24 h on cell culture medium in continuous agitation, at the same testing concentrations, was also evaluated (see chapter 3, section 3.3. Test compound preparation). Physicochemical studies conducted by the Group of Analytical Spectroscopy and Sensors of the University of Zaragoza using Single Cell Inductively Coupled Plasma Mass Spectrometry indicate that most of the silver (Ag) released from the material in *in vitro* conditions was in the form of Ag+ ions, being the amount of AgNPs released very small; more than 99% were dissolved forms of Ag(I) with less than 0.1% of AgNPs (data not published).

In this regard, it is important to emphasize in the lack of protocols adapted to NMs testing (Doak et al., 2012; EFSA Scientific Comittee et al., 2018; Evans et al., 2017; Guo and Chen, 2015; Kroll et al., 2009; Pfuhler et al., 2013; Stone et al., 2009). The rapid growth of NPs use in industry calls for a review of the current methods to correctly evaluate these materials. For example, both guidelines for the MLA (OECD TG 490) and the MN test (OECD TG 487) state that although specific adaptations for NMs may be needed, they are not described in the guidelines (OECD, 2015, 2014a). In the absence of guidelines adapted to NPs, these are the guidelines that were followed in the *in vitro* evaluation of this doctoral thesis.

2.2. Genotoxicity

As evidenced in the bibliographic revision carried out in this thesis, AgNPs can exert primary DNA damage, gene mutations and chromosomal aberrations (see chapter 4, section 1. Bibliographic revision of the genotoxicity of silver nanoparticles). However, kaolin is considered as an inert material and studies about its potential genotoxicity are very scarce. As mentioned before (see chapter 1, section 7. Toxicity of kaolin), Li and colleagues (2010) evaluated the genotoxicity of nano-silica platelets nano-silica platelets obtaining negative results in the standard comet assay, MN test and the Ames test. Surprisingly, positive responses in the *in vitro* MN test and comet assay, and the *in vivo* comet assay were obtained when evaluating micro and nano-kaolin particles (Kato et al., 2017; Kawanishi et al., 2020; Totsuka et al., 2009).

According to our knowledge, there is no information available about the *in vitro* genotoxicity of silver-kaolin based materials. Anyway, the information revised, suggested the potential reduction of the AgNPs toxicity when combining them in silver-based clay materials. Thus, the silver-kaolin formulation genotoxicity evaluation was essential.

The first phase of the silver-kaolin formulation genotoxicity evaluation was the *in vitro* testing as suggested by the EFSA strategy (Figure 2). The *in vitro* genotoxicity evaluation includes two assays to detect both gene mutations and chromosomal aberrations (EFSA Scientific Comittee et al., 2018). The usually recommended test for the evaluation of the induction of gene mutations is the Ames test, which is a well-founded genotoxicity test for chemicals, however it does not seem suitable for NPs (Butler et al., 2015; Doak et al., 2012; OECD, 2014c; Pfuhler et al., 2013). The prokaryote experimental system of the Ames test does not perform endocytosis and the bacterial wall prevents the NPs diffusion; furthermore, it is important to highlight that some NPs as AgNPs exert antimicrobial activity (Doak et al., 2012). A review performed by Lansiedel and colleagues (2009) reported positive results in bacterial gene mutation assays only in water-soluble nanoparticles suggesting the incapacity of the non-soluble NMs to cross through the bacterial cell wall. Interestingly, although AgNPs are negative for mutagenicity in the Ames test, they have been found to show positive genotoxic responses in other *in vitro*

genotoxicity assays as MLA, MN test and comet assay, being the comet assay the most used one and the MN test the second one (Azqueta and Dusinska, 2015; Guo et al., 2016; Kim et al., 2019; Landsiedel et al., 2009; Rodriguez-Garraus et al., 2020).

Among the *in vitro* mammalian cell gene mutation tests suggested by the EFSA guideline (2018), the one selected to evaluate the induction of point mutations was the MLA. The MLA is one of the most common mammalian cell assays used as an alternative to Ames test for assessing gene mutations of NMs (Doak et al., 2012; EFSA Scientific Comittee et al., 2018). Furthermore, this is the only assay used in the studies retrieved in the bibliographic revision (see chapter 4, section 1. Bibliographic revision of the genotoxicity of silver nanoparticles) for the evaluation of gene mutations. Besides, for the evaluation of chromosomal aberrations the EFSA guideline recommends the MN test (EFSA Scientific Comittee et al., 2018).

One of the possible mechanisms of AgNPs cellular toxicity is the production of oxidative stress through the generation of ROS which cause, among other effects, oxidative DNA damage (Dobrzyńska et al., 2014; McShan et al., 2014). DNA base oxidation is the most common DNA damage caused by NPs, since increased ROS levels have been observed in several studies (Pattan and Kaul, 2014). Although not yet validated, to assess oxidative DNA damage and to provide complementary information about the genotoxic mechanisms of NPs, the EFSA guideline recommends carrying out the enzyme-modified *in vitro* comet assay (EFSA Scientific Comittee et al., 2018). In this work the Fpg-modified comet assay was used to detect oxidize bases, though it can also detect other lesions (Azqueta et al., 2013; Muruzabal et al., 2021). Thus, the *in vitro* evaluation was complemented by the also recommended enzyme-modified comet assay (EFSA Scientific Comittee et al., 2018).

Therefore, the *in vitro* genotoxicity strategy composed by 1) the MLA for the evaluation of point mutations, 2) the MN test for the evaluation of chromosomal aberrations and 3) a complementary standard and Fpg-modified comet assay for the evaluation of DNA base oxidation, was conducted (Figure 7). The MLA and the MN test were performed following the principles of their corresponding OECD guidelines, using L5178Y $TK^{+/-}$ and TK6 cells, respectively (OECD, 2015, 2014b). The comet assay was conducted using TK6 cells. All the *in vitro* assays were carried out without metabolic activation system (S9) since poorly soluble NMs are not metabolized by S9 and moreover, it may interfere with the assay reducing the NM bioavailability (EFSA Scientific Comittee et al., 2018; Pfuhler et al., 2013; Xu et al., 2012). All tests were carried out under GLPs-like conditions.

2.2.1. Cytotoxicity and proliferation assays

First, in order to have preliminary results for the selection of the silver-kaolin concentrations to be tested on each *in vitro* assay, cytotoxicity and proliferation assays were conducted both with the silver-kaolin formulation and its release (Figures 10 and 11). The results of the cytotoxicity and proliferation assays carried out with the

silver-kaolin formulation helped to decide the concentrations to be tested in the cytotoxicity and proliferation assays for silver-kaolin release and for each of the *in vitro* genotoxicity assays. The survival % and the RSG % were considered as affected when results were lower than 80%.

The cytotoxicity in both cell lines may be caused by a combination of physical damage produced by the collision of the suspended particles in the medium with the cells and the damage produced by the silver-kaolin release. Treatments were carried out using gently shaking and as silver-kaolin formulation is not soluble, particles were in continuously movement mixed in the suspension and hitting the cells. In addition, considerable SDs were observed in some cases (Figures 10 and 11), that may be due to the complex composition of the test material.

The maximum concentrations tested in MLA and MN test were established following the corresponding OECD guidelines; these concentrations need to exert a certain level of toxicity in the specific toxicity test that accompanies these assays (see next sections). On the other hand, the *in vitro* comet assay does not have an OECD guideline and thus it was important to establish concentrations which showed relevant results in this assay, i.e., testing non-toxic concentrations (RSG > 80%) (Collins et al., 2017; Huk et al., 2015, 2014; Yamani et al., 2017), testing both the silver-kaolin formulation and silver-kaolin release, which resulted less cytotoxic. However, an adequate number of concentrations covering a wide range of toxicity were tested in all three assays.

2.2.2. Mouse lymphoma assay

The MLA was carried out using L5178Y $TK^{+/-}$ cells. The induction of gene mutations was evaluated after 3 h and 24 h treatment with silver-kaolin formulation and its corresponding release. According to the OECD TG 490, the maximum concentration to be tested in the MLA should reach a 10% RTG if based on cytotoxicity, meaning that the highest dose should induce a 90% cytotoxicity. When no limiting cytotoxicity is observed, the maximum concentration to be tested should be 10 mM, 2 mg/mL or 2 µl/mL, whichever is the lowest (OECD, 2015).

The most appropriate concentration to be tested in order to achieve a 10% RTG, would have been 10 mg/mL, but it was too high in comparison to the guideline recommendations (OECD, 2015). For this reason, the maximum concentration tested in the MLA 3 h treatment was 3.33 mg/mL. The maximum concentration tested in the MLA 24 h treatment was 2 mg/mL, the maximum recommended by the guideline.

The cytotoxicity results obtained in the MLA did not exactly agree with those of the previous proliferation assays, probably due to the different parameter evaluated. In the MLA, the cytotoxicity study includes the proliferation of the cells throughout the up to 14 days the assay lasts, expressed as RTG %. In contrast, the measurement

of the preliminary proliferation tests only considers the cells until proliferation 48 hours after treatment, expressed as RSG %.

The acceptability of the MLA was evaluated applying the OECD TG 490 recommendations (OECD, 2015). Regarding negative control, MF must range between 50 and 170 $x10^{-6}$, CE must range between 65 and 120% and TSG must range between 8 and 32-fold for short treatment, and between 32 and 180-fold for long treatment (OECD, 2015). In relation to the positive control, it must show an absolute increase in MF of at least $300x10^{-6}$ above negative control, being at least a 40% of the colonies small ones. In the present study, the negative control MF values were $60.9x10^{-6}$ and $163x10^{-6}$ for 3 h and 24 h respectively, CE values were 71.4% and 77% for 3 h and 24 h, respectively and TSG values were 28.1 and 78.3 for 3 h and 24 h, respectively (Figure 12). Regarding positive control, MF values were $431x10^{-6}$ for 3 h treatment and $946x10^{-6}$ for 24 h treatment, being the total of colonies small ones (Figure 12). Thus, all these values met the criteria of the guideline. In the MLA, a response is considered as positive if any of the experimental conditions shows a MF higher than the MF of the negative control plus $126x10^{-6}$ and if there is a concentration-dependent increase in MF. None of the silver-kaolin formulation or its corresponding silver-kaolin release concentrations tested, at any of the treatment times, showed an increase in the MF higher than their respective negative control plus $126x10^{-6}$ (Figure 12). Furthermore, there was not any concentration-related increase in MF at any of the treatment times with either silver-kaolin formulation or its corresponding release. Given the clearly negative results, there was no requirement for verification (OECD, 2015).

2.2.3. Micronucleus test

The MN test was carried out using TK6 cells. The induction of chromosomal aberrations was evaluated after 3 h treatment with silver-kaolin release and after 24 h treatment with silver-kaolin formulation and its corresponding release. According to the OCED TG 487, the maximum concentration to be tested in the MN should achieve a 55±5% cytotoxicity and when no limiting cytotoxicity is observed, the maximum concentration to be tested should be 10 mM, 2 mg/mL or 2 µl/mL, whichever is the lowest (OECD, 2014b).

For 3 h treatment the maximum concentration tested was 2 mg/mL, following the guideline recommendations; however, only the silver-kaolin release was tested due to the impossibility of eliminating the silver-kaolin formulation after 3 h of treatment, which would turn it into a 24 h treatment. For 24 h treatment, the maximum concentration tested was 0.5 mg/mL, showing a 30% RSG in the proliferation assays. The cytotoxicity study of the MN test considers the number of living cells at the end of the 1.5 cycles the test lasts. Furthermore, the tests results were analysed by flow cytometry and cytotoxicity was measured by adding a known number of beads. For this reason, the cytotoxicity results of the MN tests did not exactly agree with those of the preliminary cytotoxicity assays. Moreover, the results were very variable between duplicates.

The acceptability of MN tests was evaluated applying the OECD TG 487 criteria: negative and positive controls were compatible with the historical control data of the toxicology laboratory and positive controls produced a significant increase in response with respect to negative control (OECD, 2014a). As MN were assessed by flow cytometry, results were considered as positive based on three criteria: 1) a statistically significant increase in the MN/1000 N compared with the negative control, in at least one of the concentrations tested, 2) a three-fold increase in MN at one or more concentrations over the negative control, 3) a concentration-related MN increase over the non-cytotoxic range tested (i.e., RS > 40%). Although some of the total MN data were statistically significant in comparison to the negative control, no testing concentration of the compound and its release, induced a three-fold increase in MN over the negative control and furthermore, there was no concentration-related response (Table 11). According to the OECD TG 487, a clearly negative result does not need verification (OECD, 2014a). However, the OECD guideline is focused on the analysis of MN on slides and not by flow cytometry. Thus, a confirmatory experiment was performed, and these results were confirmed.

2.2.4. Standard and Fpg-modified comet assay

The standard and Fpg-modified comet assay was carried out using TK6 cells. The induction of SBs, ALS and oxidized bases, was evaluated after 3 h and 24 h treatment with silver-kaolin formulation and its corresponding release. The maximum concentrations tested were 1.5 mg/mL and 0.5 mg/mL for 3 h and 24 h, respectively. Concentrations covering a wide range of toxicity, including two toxic (RSG < 80) and three non-toxic (RSG > 80) concentrations for each treatment time, were tested. This was performed with the aim to achieve high concentrations of released AgNPs/Ag^{+}, in order to evaluate their possible induction of oxidation. In any case, an adequate number of concentrations covering a wide range of toxicity were tested. None of the concentrations tested of the compound and its release, in any of the conditions tested, showed significant differences in the level % DNA in tail for ALS and SBs, or Fpg-sensitive sites, compared to the negative control. Since the *in vitro* comet assay does not have an OECD guideline, three independent experiments were carried out and analysed together.

2.3. General considerations

To correctly interpret NPs negative results, it is necessary to take into account whether the material tested has been in contact with the cells or not (EFSA Scientific Comittee et al., 2018; International Organization of Standarization, 2017). As previously mentioned, the silver content (internalized or adsorbed by the cells) of individual cells was determined by mass distribution obtained by Single Cell Inductively Coupled Plasma Mass Spectrometry (see chapter 3, section 2: test compound). The technique couldn't distinguish between dissolved silver and silver nanoparticles inside the cells, but the direct contact between cells and silver was evidenced (Figure 6).

Both the silver-kaolin formulation and its corresponding silver-kaolin release tested in the *in vitro* assays of this study showed clear negative results in all of them. Considering only the AgNPs content of the silver-kaolin formulation, the concentrations tested in each of our assays corresponded to the following ones:

Table 23. Ranges of silver-kaolin formulation concentrations tested on each *in vitro* assay and their corresponding AgNPs content.

Assay	Treatment time (h)	Silver-kaolin formulation (mg/mL)	AgNPs (µg/mL)
MLA	3	0.12-3.33	0.1-27.6
	24	0.07-2	0.58-16.6
MN	24	0.02-0.5	0.17-4.15
Comet	3	0.02-1.5	0.17-12.45
	24	0.01-0.5	0.083-4.15

It must be noted that the AgNPs concentrations that were in contact with the cells in the *in vitro* studies carried out were low (Table 21). However these concentrations correspond to some of the ones tested in the studies reviewed in the bibliographic revision, which showed positive results (see chapter 4, section 1. Bibliographic revision of the genotoxicity of silver nanoparticles). Furthermore, some of the positive results in MLA and MN assays corresponded to AgNPs tested on L5178Y $TK^{+/-}$ and TK6 cells (Guo et al., 2016; Y. Li et al., 2017). This may suggest that the AgNPs attachment to kaolin decrease their toxicity, in accordance with other studies with similar materials (Su et al., 2012, 2012). It should be noted that the material was not completely removed after washing in any of the MLA or MN tests (24 h treatment), so it was present in all the corresponding post-treatment incubations, which makes the negative results even more significant. Furthermore, it is important to highlight that the silver-kaolin formulation demonstrated its *in vitro* antimicrobial activity at concentrations much lower than the ones tested in our studies, showing minimum inhibitory concentrations of 3.9-15.6 µg/mL and minimum bactericidal concentrations of 7.8-250 µg/mL, against several bacterial strains (Pérez-Etayo et al., 2021).

Negative results in *in vitro* studies do not usually require an *in vivo* follow up, but regarding this type of complex material, the *in vivo* evaluation is advisable for a correct evaluation of the safety, since the *in vitro* assays have some limitations and the OECD guidelines are not yet adapted (Doak et al., 2012; EFSA Scientific Comittee, et al., 2018; Evans et al., 2017; Guo et al., 2016). Moreover, some NMs are known to cause inflammatory effects, leading to the generation of ROS and triggering secondary genotoxicity, which cannot be detected by *in vitro* systems (EFSA Scientific Comittee et al., 2018; Magdolenova et al., 2014; Stone et al., 2009). For this reason, silver-kaolin formulation safety evaluation was continued by conducting an *in vivo* evaluation.

3. *In vivo* toxicity and genotoxicity evaluation of silver-kaolin formulation evaluation

According to the EFSA guidance, the suitable *in vivo* tests for NMs genotoxicity evaluation are the *in vivo* micronucleus test (OECD, 1997b), the *in vivo* mammalian alkaline comet assay (OECD, 2016a) and the transgenic rodent somatic and germ cell gene mutation assay (OECD, 2011), being advisable the combination of these tests applied to the same individual animals (EFSA Scientific Comittee et al., 2018).

The comet assay is frequently used in the *in vivo* genotoxicity evaluation of NMs (Azqueta and Dusinska, 2015; Gliga et al., 2014; Narciso et al., 2020; Pfuhler et al., 2013); actually, it was the most used in the case of the AgNPs genotoxicity evaluation (Rodriguez-Garraus et al., 2020). The assay offers the opportunity to analyze different organs in a quite simple manner. However, lesions detected by the comet assay are reparable, thus the MN assay is a good complement to detect chromosomal aberrations. Furthermore, when conducting an *in vivo* evaluation, the EFSA guidance recommends the combination of an *in vivo* comet assay and other genotoxicity tests, such as the MN test, included in a repeated-dose oral toxicity study, to provide useful information about the secondary genotoxicity mechanisms caused by inflammation (EFSA Scientific Comittee, et al., 2018; Evans et al., 2017; Pfuhler et al., 2013).

Taking all this into account, the following *in vivo* evaluation strategy was carried out: 1) a dose finding study and 2) an *in vivo* mammalian alkaline comet assay (OECD, 2016a) and an *in vivo* micronucleus test (OECD, 1997b), both of them integrated in a repeated dose 28-day oral toxicity study in Wistar rats (OECD, 2018a) (Figure 9). All studies were carried out under GLPs-like conditions.

3.1. Dose finding study

First, and because no data of silver-kaolin formulation or other similar materials toxicity was available, a preliminary acute oral toxicity dose finding study in females was carried out following the principles of the OECD TG 425 (OECD, 2008). Data available about the toxicity of AgNPs and kaolin separately were considered for this study. A Lethal Dose 50 (LD50) greater than 5000 mg/kg b.w. in rodents had been estimated for kaolin (Agency, 2000; Maisanaba et al., 2015). Also, AgNPs with sizes between 10-20 nm and 8-20 nm have shown low toxicity both in single dose and in short-term repeated dose toxicity studies, up to 5000 mg/kg b.w. (Adeyemi and Adewumi, 2014; Maneewattanapinyo et al., 2011).

Consequently, the maximum dose to be tested in the dose finding study was decided to be 2000 mg/kg b.w. silver-kaolin formulation, that is the highest dose suggested by the OECD TG 425 (OECD, 2008). In order to have information of toxicity after a few repeated doses, a specific experimental design was decided that permitted to administer the high dose for a maximum of 7 days, to 5 animals, due to the fact that no toxicity was observed after one single dose. The only effect detected in the animals was a lower fattening than expected. This was not

considered as a relevant toxic effect because the animals didn't lose weight, but an alert was established to take into account in the repeated dose 28-day oral toxicity study.

Considering all the results together, no signs of toxicity were observed, therefore the significant specific findings were not considered relevant. Thus, 2000 mg/kg b.w. was established as the high dose for the 28-day oral toxicity study in male and female Wistar rats in which the alkaline comet assay and the *in vivo* MN test in females were integrated (OECD, 2018a, 2016a, 1997b)

3.2. Repeated dose 28-day oral toxicity study

According to the OECD TG 407 at least three test groups and a control group should be used for a repeated dose 28-day oral toxicity study. As no toxicity was observed after 7 administrations of 2000 mg/kg b.w. silver-kaolin formulation, this was the dose selected as the highest one just in case it could have any toxic effect after four weeks daily treatment. The lowest dose was determined based on an antimicrobial study of a similar formulation in piglets: a material composed by AgNPs in a sepiolite matrix showed antibacterial effects at 40 mg/kg b.w. (Fondevila et al., 2009). This dose extrapolated to the experimental system of the present study corresponds approximately to a dose of 50 mg of silver-kaolin formulation/kg b.w. in rat. Finally, the medium dose (300 mg/kg b.w.) was the geometric mean between the high and the low dose.

The 28-day oral administration of silver-kaolin formulation, at doses of 50, 300 and 2000 mg/kg did not produce mortality or strong adverse effects. Besides, the weight growth alert detected in the dose finding study was not confirmed, as the animal growth in the repeated dose 28-day oral toxicity study was in accordance with the normal ranges provided by the animal supplier (Envigo). Statistical differences were found in some analytical data; however, when analysing the individual data, it was found that most of them were within the normal ranges of the laboratory historical values. Altered parameters were not considered relevant from the toxicological point of view when all obtained data about each animal were analysed together.

In other *in vivo* studies with 125-300 mg/kg b.w. of 60 and 56 nm AgNPs orally administered to rats for 28 or 90 days respectively, some significant dose-dependent changes in the ALP and cholesterol values in either male or female rats, were observed; thus, these AgNPs range of doses might result in slight liver damage (Kim et al., 2008; Park et al., 2010). By contrast, 20 nm AgNPs at doses of 50-300 mg/kg b.w. administered orally for 3 days showed accumulation in duodenum, blood, liver, spleen and kidneys, but no toxic effects were detected in those tissues (Narciso et al., 2020). In the same way, oral administration of 10, 75, and 110 nm AgNPs at 9-36 mg/kg b.w. for 28 days did not show relevant toxic effects in rats (Boudreau et al., 2016). The silver-kaolin formulation of the present study contained approximately a 1 % AgNPs, and consequently it can

be estimated an intake of 0.5-20 mg/kg b.w. of AgNPs. Therefore, the results obtained in this study are in accordance with the low toxicity detected by Bodreau et al. (2016) at equivalent doses of AgNPs.

Furthermore, the oral administration of silver ions at high doses (400 mg/kg b.w.) for 28 days produced a high morbidity in rats, consisting in severe gastrointestinal effects (Boudreau et al., 2016). Considering the low rate of the Ag^+ released from the silver-kaolin formulation previously mentioned, the Ag^+ concentration would be much lower than 400 mg/kg b.w., which could explain the absence of toxicity. In any case, it must be taken into account that these release studies were carried out in *in vitro* conditions, so they do not fully conform to the reality of *the in vivo* results.

On the other hand, only one study regarding kaolin toxicity has been found and it is worth to note that is a very old study. After a single oral administration of 1000 to 2100 mg kaolin/kg b.w., toxic effects as listlessness, anorexia, oliguria, hypothermia, and dyspnea, due to the accumulation, obstruction and perforation of the alimentary canal bowel were detected (Biology, 1977). In contrast, in our 28-day repeated dose oral toxicity study, doses up to 2000 mg/kg b.w. did not produce any similar toxic effects.

3.3. Genotoxicity

All samples evaluated in the *in vivo* genotoxicity studies were obtained from the repeated dose 28-day oral toxicity study in female Wistar rats, carried out following the principles of the OECD TG 407 (OECD, 2018a). This experimental design was established in accordance with the principles of the 3Rs (Replacement, Reduction and Refinement) as the same animals were used for the toxicity and the genotoxicity evaluation. Moreover, as stated in the OECD TG 489 and OECD TG 474, testing in one single sex is enough for the evaluation (OECD, 2016a, 1997b).

The choice of only females in the genotoxicity studies was based on bibliographic evidence of a gender related AgNPs accumulation. After 13 weeks oral administration of 10, 75 and 100 nm AgNPs at 9- 36 mg/kg b.w., higher accumulation was observed in the kidney, liver, jejunum and colon of female rats compared to males (Boudreau et al., 2016). Moreover, after 28-day oral administration of 60 nm AgNPs, to Sprague Dawley rats, at doses of 30, 300 and 1000 mg/kg b.w., a two-fold higher accumulation was observed in kidney of females when compared to males (Kim et al., 2008). This effect was confirmed a year later, after the administration of the same 60 nm AgNPs, to Fischer rats for 13 weeks, showing higher accumulation in all regions of female kidneys compared to male kidneys (Kim et al., 2009).

3.3.1. Micronucleus test

The MN test was carried out with samples of femur bone marrow. The acceptability of the MN test was evaluated applying the OECD TG 474 recommendations (OECD, 1997b). The MN frequencies obtained for the positive and the negative controls were those expected considering the data generated in the laboratory in previous studies. Mitomycin C at a dose of 4 mg/kg b.w. promoted much higher induction of MN than the vehicle of the test product (negative control) and the statistical comparison of these values determined the existence of very significant differences ($p = 0.009$).

None of the results obtained for all silver-kaolin formulation doses, showed a statistically significant increase in MN in comparison to the negative control, and a dose related response was not observed. Taking all this into account, the MN results of this study were considered as clearly negative, i.e., this compound does not induce chromosomal aberrations under the conditions tested. We could not demonstrate bone marrow exposure to the test product but there is bibliographic evidence proving that AgNPs or the silver contained in them can reach blood circulation and affect bone marrow, after oral administration (Kim et al., 2008; Van Der Zande et al., 2012).

3.3.2. Standard comet assay

Liver, kidneys, spleen and duodenum were chosen as target organs for the comet assay as they showed a notable accumulation of AgNPs in previous studies carried out in rodents after repeated administrations (Boudreau et al., 2016; Jiménez-Lamana et al., 2014; Kim et al., 2009, 2008; Narciso et al., 2020). After the 28-day oral administration of AgNPs ranging from 15 nm to 60 nm, they were detected in gastrointestinal tract, liver, kidney and spleen a day after the last administration (Kim et al., 2008; Loeschner et al., 2011; Van Der Zande et al., 2012). Furthermore, a week after the last administration, AgNPs remained accumulated in kidneys and spleen, and even 8 weeks after the last dose, in spleen (Van Der Zande et al., 2012). After a single intravenous dose of 5 mg/kg b.w. of AgNPs of 20 or 200 nm, different pattern of biodistribution was found between the target organs (Dziendzikowska et al., 2012). Liver was the main accumulation site 24 h after the dose; then, 7 days after the administration, a higher level of silver was found in spleen, and finally, 28 days after the administration, silver was mainly accumulated in kidneys.

Duodenum was also selected as a site-of-contact tissue since the exposure route to silver-kaolin formulation was oral (Fondevila et al., 2009; Food and Authority, 2012; Jerome, 2012; Kirkland et al., 2019; OECD, 2016a). Information on the distribution of kaolin is very scarce. Although some studies have evidenced the dissociation of kaolin in the gastrointestinal tract, leading to the distribution and accumulation of aluminum in kidney, liver, heart and brain, in other cases, the accumulation of this metal has been undetectable (reviewed in Maisanaba et al., 2015).

The acceptability criteria of the standard comet assay were those recommended in the OECD TG 849 guideline. The % DNA in tail values obtained for the negative and positive controls were those expected, considering the data generated in the laboratory in previous studies. Regarding the control group, the OECD TG 489 suggests the mean % DNA in tail in rat liver should not exceed a 6 % (OECD, 2016a). In this regard, the value obtained for the negative control group was 4.09 ± 1.66 %. The results showed little variability, except for two values in kidney and one in duodenum that were much higher than the others (Figure 18). It is worth mentioning that the tissues with high values did not come from the same animals.

Regarding the positive control, MMS at a dose of 200 mg/kg b.w. induced a much higher level of DNA damage than the distilled water (negative control); the statistical comparison of these values determined the existence of very significant difference ($p = 0.009$).

None of the results obtained for all silver-kaolin formulation doses and tissues showed a statistically significant increase in % DNA in tail, in comparison to their corresponding negative control and a dose related response was not observed. Thus, the results were considered as clearly negative, i.e., the presence of DNA SBs or ALS was not detected.

3.3.3. Fpg-modified comet assay

The Fpg-modified comet assay does not have an OECD guideline. The levels of Fpg-sensitive sites obtained in the negative control group were in concordance with the previously obtained by the laboratory. Also, the level of Fpg-sensitive sites obtained in the assay controls was as expected (i.e., 67.30 ± 9.30 %), which indicated the good performance of the assay.

A possible toxicity mechanism of AgNPs is the oxidation, reason why the EFSA guidance recommends including the modified comet assay in the genotoxicity evaluation (Dobrzyńska et al., 2014; EFSA Scientific Comittee, et al., 2018; McShan et al., 2014). It should be noted that the inclusion of the Fpg-modified comet assay was also performed according to the principles of the 3Rs. In this case, a positive control itself was not used to reduce the number of animals in the study. For this reason, assay controls were included as positive control for each Fpg-comet assay, consisting in TK6 cells treated with $KBrO_3$. Nevertheless, the MMS induces both SBs and Fpg-sensitive sites, and it can be used as a positive control for the standard comet and the Fpg-modified comet assay (Enciso et al., 2018). However, different doses of MMS are needed to induce DNA SBs and Fpg-sensitive sites. It would be interesting to conduct studies to find out if there is an MMS dose which induces detectable levels of both types of lesions.

An induction of Fpg-sensitive sites was not observed at the highest dose, thus oxidized bases were not detected under the conditions tested.

3.4. General considerations

As far as we know, there are no genotoxicity studies of AgNPs-kaolin based materials, but it is known that AgNPs have the capacity to induce chromosomal aberrations and to oxidize DNA bases *in vivo* (Tables 6 and 8). Regarding the studies in which the AgNPs have also been administered orally and at similar schedules, AgNPs of 5 nm, orally administered to mouse once a day, for 35 days, at doses of 10 to 20 mg/kg b.w., resulted positive in comet assay (Kant Awasthi et al., 2015). AgNPs of 20 nm, orally administered to mouse, once a day, for 28 days, at doses of 10 to 250 mg/kg b.w. induced a significant increase of MN in bone marrow (Wang et al., 2019). The results of the present study are negative, but the AgNPs exposure dose is unknown and probably much lower. As explained before, assuming a complete nanoparticles release from the kaolin matrix, the maximum dose of AgNPs would be 20 mg/kg b.w. (see chapter 4, section 3.2. Repeated dose 28-day oral toxicity study). Furthermore, as mentioned before, only a minimal fraction of the AgNPs embedded in the kaolin matrix is released. Therefore, the dose of AgNPs that would be available could be even lower.

On the other hand, only two *in vivo* genotoxicity assays have been carried out with kaolin, showing its ability to induce SBs in lung tissues after intracranial instillation (Kato et al., 2017; Totsuka et al., 2009). The comparison of these results with those obtained in this study is very complicated since both the route of administration and the target organ are different.

According to our knowledge, safety studies of other silver-kaolin based materials have not been carried out. Nonetheless, some safety evaluations of other materials composed by different clays as carriers of AgNPs have been conducted, concluding that clays have very low toxicity and suggesting that the immobilization of AgNPs onto their surfaces reduced silver accumulation in some tissues and improved the safety of AgNPs, while their antimicrobial activity remained (Su et al., 2012). The *in vivo* acute toxicity of a material composed by nano-silica platelets (NSP) and AgNPs (AgNPs-NSP) was evaluated by Chiao and colleagues (2012). One-day old chicks were feed with either 500 mg/kg b.w. AgNPs-NSP, 500 mg/kg b.w. NSP or 187.5 mg/kg b.w. AgNPs. Neither of the three groups showed notably toxic effects in tissues and furthermore, gastrointestinal epithelial cells remained intact, and no inflammatory cells were detected. In the same study, curious results were found in the silver distribution. As expected, silver levels in the tissues of NSP-feed group were very low and silver levels in gastrointestinal tract were significantly higher in AgNPs-feed group than in AgNPs-NSP-feed one. By contrast, silver levels in liver and kidney were high and similar in both groups feed with AgNPs and AgNPs-NSP. This may be because the free AgNPs accumulate in the gastrointestinal tract, instead only the Ag ions are absorbed reaching other tissues through blood circulation. Therefore, regarding AgNPs-clay materials, the

AgNPs seem to remain attached to the clays surface while the released Ag ions are absorbed. This could also occur with the silver-kaolin formulation of the present study. It also may be one of the reasons why toxic or genotoxic effects of the silver-kaolin formulation were not observed with respect to the known toxic effects of AgNPs.

4. Future perspectives

Finally, in order to correctly interpret the results obtained for the silver-kaolin formulation, it would be interesting to conduct a biodistribution study of silver-kaolin formulation, and the AgNPs and kaolin separately. In this way, the absorption, distribution to target organs and accumulation could be observed for each material independently. In addition, it could be determined if the combination of both kaolin and AgNPs had any effect on the AgNPs biodistribution or toxicity. All of this could be conducted, following the next stage of the strategy recommended by the EFSA guideline (EFSA Scientific Comittee et al., 2018) by carrying out a modified repeated dose 90-day oral toxicity study in rodents, including a satellite group for the assessment of absorption, tissue distribution and accumulation (OECD, 2018b).

Chapter 6. Conclusions

With regard to the bibliographic revision of the genotoxicity of silver nanoparticles (objective 1):

1. Positive results were obtained for every damage level (i.e., primary DNA damage, gene mutations and chromosomal aberrations), being predominant in the *in vitro* assays.
2. There is a lack of a comprehensive evaluation of AgNPs genotoxicity through a complete battery of assays including both *in vitro* and *in vivo* experimental designs, as recommended in the EFSA strategy. Furthermore, OECD guidelines were rarely used.
3. Few *in vitro* and *in vivo* studies carried the enzyme-modified comet assay however, results indicate a possible oxidative mechanism. This mechanism needs further evaluation.

With regard to the evaluation of the *in vitro* genotoxicity of silver-kaolin formulation (objective 2):

4. The methods established for cells washings after treatment were decantation for MLA and centrifugation for MN test and comet assay.
5. Silver-kaolin formulation didn't induce gene mutations or chromosomal aberrations.
6. Silver-kaolin formulation didn't induce strand breaks, alkali labile sites or oxidized bases.

With regard to the evaluation of the *in vivo* toxicity and genotoxicity of silver-kaolin formulation in rats (objective 3):

7. Silver-kaolin formulation at 2000 mg/kg b.w. was established as the maximum dose for the 28-day oral toxicity study. The other doses selected were 50 mg/kg b.w. as the efficacy dose and 300 mg/kg b.w. as the intermediate dose.
8. The no observed adverse effect level (NOAEL) of silver-kaolin formulation was determined to be a dose of 2000 mg/kg b.w. by oral route, for 28 days.
9. Silver-kaolin formulation didn't induce chromosomal aberrations in bone marrow of female rats after 28-day oral administration at any dose.
10. Silver-kaolin formulation didn't induce DNA SBs or ALS in liver, spleen, kidney or duodenum of female rats after 28-day oral administration at any dose.
11. Silver-kaolin formulation didn't induce oxidized bases in liver, spleen, kidney or duodenum of female rats after 28-day oral administration at doses of 2000 mg/kg b.w.

After the current genotoxicity evaluation, no concerning effects of silver-kaolin formulation have been observed.

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Scientific dissemination

Publications

- Rodriguez-Garraus A, Azqueta A, Vettorazzi A, López de Cerain A. Genotoxicity of Silver Nanoparticles. NMs (Basel). 2020 Jan 31;10(2):251. doi: 10.3390/nano10020251.

Congresses

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