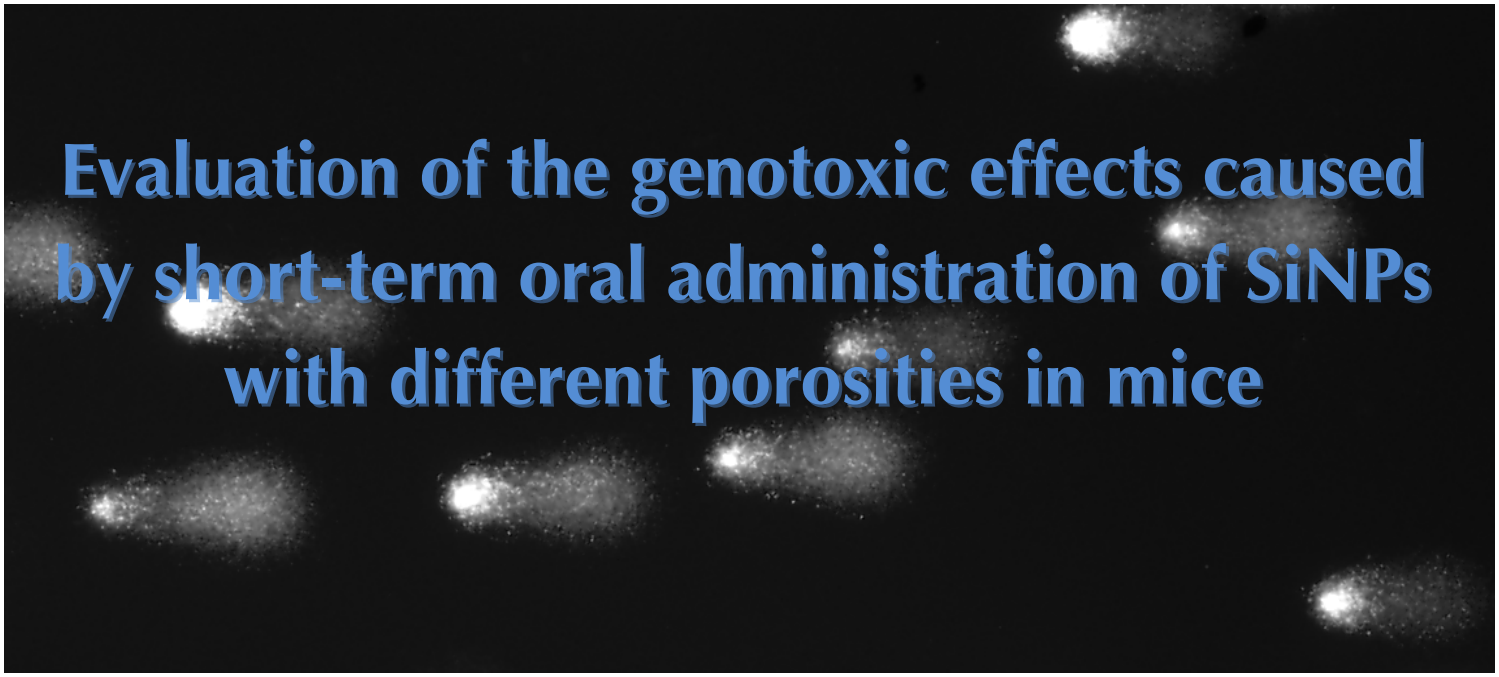

Evaluation of the genotoxic effects caused by short-term oral administration of SiNPs with different porosities in mice

Irene Gimeno Benito

Nanobiotechnology Master Thesis

2017 - 2018

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Silicon oxide nanoparticles (SiO_2 NPs), also known as silica nanoparticles (SiNPs) are being applied in different fields, such as cosmetics, food industry or biotechnology. Amorphous silica powder is widely used in food industry as an anti-caking agent (E551). For that reason, the general population is exposed to E551 that is based on micro-sized aggregated of nano-sized primary particles. With the emerging application of new products in the food industry, it is necessary to assess the risks associated to the use of specific nanomaterials.

In this study, the short-term local genotoxicity of SiNPs with different porosities has been tested in mice by the oral route. The evaluation of DNA damage has been performed in jejunum samples of mice exposed to four different SiNPs with different sizes (100 nm and 300 nm) and porosities (porous and non-porous) at two doses (100 mg/kg and 1000 mg/kg) and at two time points (at day 6 and at day 26).

The single cell gel electrophoresis, most commonly known as alkaline comet assay, has been used to evaluate and quantify DNA damage (genotoxicity) *in vivo*. This assay is a versatile and sensible technique compared to other genotoxic tests.

The results showed that none of the NPs tested showed an increase in % of tail intensity when compared to the vehicle control group at day 6 nor at day 26. These results lead us to conclude that after 5 days of oral gavage, none of the tested nanoparticles generated detectable genotoxic effects in jejunum by the comet assay.

PREFACE

This report has been written by Irene Gimeno Benito during the 9th and 10th semester as a Long Term Master Thesis from the Nanobiotechnology Master Program from Aalborg University. The thesis is based on a research that has been developed entirely at the Human Health Safety Group (HEHS) from LEITAT Technological Center in Barcelona. It has been produced in the period starting from September the 1st of 2017 to June the 1st of 2018. The subject of the report is the evaluation of the genotoxic effects caused by short-term oral administration of SiNPs with different porosities in mice.

Reading Guide

Throughout the report, there will be references to various sources. These will be found on the form (Author, Year of publication) where the information inside the brackets refers to the author of a specific source in the bibliography at the end of the report. Tables and figures are listed after the number of the section in which they are displayed as *Figure#* or *Table#*. To each table or figure a short descriptive caption will be made together with a bibliographic reference. If there is no reference in a caption, the figure is produced by report's author. Abbreviations will be found in the next page.

Irene Gimeno Benito

Barcelona, 2017 - 2018

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ABBREVIATIONS

CMC: Carboxymethyl cellulose

DMSO: Dimethyl sulfoxide

DSB: Double-Strand Breaks

EDTA: Ethylenediaminetetraacetic acid

EMS: Ethyl Methane Sulfonate

ENM: Engineered Nanomaterials

ENPs: Engineered Nanoparticles

FPG: Formamidopyrimidine DNA-glycosylase

HBSS: Hanks' Balanced Salt Solution

LMPA: Low Melting Point Agarose

MMS: Methyl Methane Sulfonate

MN: Manufactured Nanoparticles

MØ: Macrophage

NM: Nanomaterial

NP: Nanoparticle

Ø: Diameter

PBS: Phosphate Buffer Saline

RCS: Respirable Crystalline Silica

ROS: Reactive Oxygen Species

SAS: Synthetic Amorphous Silica

SCGE: Single Cell Gel Electrophoresis

SEM: (microscopy) Scanning Electron Microscopy

SEM: (statistics) Standard Error Mean

SiNPs: Silica Nanoparticles

SSA: Specific Surface Area

SSB: Single-Strand Breaks

Tris: Tris(hydroxymethyl)aminomethane

WP: Work Package

1. INTRODUCTION

1.1. State of the Art

Nanomaterials are widely used in many areas due to their unique physical and chemical characteristics. There is an enormous interest of applying engineered nanomaterials (ENM) in different fields such as ceramics, catalysts, micro-electronics, coatings and foodstuffs among others. Silicon (28% by mass) is the second most abundant element on the Earth crust after oxygen. In this scenario, it is not a coincidence that silicon dioxide (silica, SiO_2) is one of the most abundant compounds in nature. It exists in amorphous and crystalline forms. Silica is generally used in such products as varnishes, drug additives and food ingredients in its bulk form.

Synthetic amorphous silica (SAS) nanoparticles are being applied in different biotechnological areas like cosmetics (Zhu *et al.*, 2010), drug delivery therapies (Bottini *et al.*, 2007), food industry or chemical industry. Nanosilica is widely used as anti-caking agent in food industry (E551) and therefore the general population is very exposed to it. However, its potential hazard effect at the gastrointestinal tract is still on research.

It has been proved that materials can be inactive in bulk form but might be toxic at the nanoscale (Bergamaschi *et al.*, 2006). For that reason, it is an important issue to evaluate the human and environmental toxic effects that they can produce. Due to their small size, nanoparticles (NPs) could easily enter into the human body via diverse routes such as dermal absorption, ingestion or inhalation. It has been demonstrated that NPs can be deposited in the lungs inducing inflammation, fibrosis and cytotoxicity when they are inhaled (Oberdörster *et al.,* 2005).

Toxicology refers to the adverse effects that some chemicals can produce to humans, animals or to the environment. While some materials are innocuous in their bulk form, when they are made into smaller and smaller pieces their surface properties changes thus, increasing their chemical reactivity (Jefferson, 2000). The toxic potential of nanomaterials is being investigated trying to catch up with the rapid growth of nanotechnology.

The property of a certain substance to produce a toxicological response at the DNA level is known as genotoxicity. Modifications in the genetic material can lead to cancer generation and, for that reason, it is very relevant to evaluate if a substance has the ability to produce harmful effects in DNA. Genotoxicity can be tested either *in vitro* or *in vivo* with different tests. The most common genotoxicity tests are the alkaline comet assay (OECD TG 489, 2014), the micronucleus assay (OECD TG 487, 2014) and the chromosome aberration assay (OECD TG 473, 1997).

The comet assay is also known as Single Cell Gel Electrophoresis (SCGE) and it is used to detect and quantify DNA damage in individual cells. It was initially proposed in 1984 by Ostling and Johanson (1984) and then modified in 1988 by Singh and colleagues (1988) who suggested to perform the assay under alkaline conditions. The method is based on the obtainment of cell suspensions which are subjected to different treatments including

cell lysis, relaxation of DNA supercoils (unwinding) and an electrophoresis. The DNA damage pattern is visualized as a comet after the electrophoresis. The tail of the comet provides information about the level of damage.

The cytotoxic and genotoxic effects of several types of nanoparticles and fine particles relevant as food additives have been investigated by Gerloff *et al*. (Gerloff *et al.*, 2009) on intestinal Caco-2 cells. They have proven that all particles, except for MgO, were cytotoxic; and that ZnO, and to a lesser extent SiO_2, induced significant genotoxicity. SAS nanoparticles are demonstrated to induce reactive oxygen species (ROS) and DNA damage leading to cytokine release and apoptosis in macrophage cells (Hamilton, Thakur and Holian, 2008).

Tarantini *et al.* (2015) studied the *in vivo* genotoxicity of four different SAS nanoparticles in rats following oral exposure. Rats were exposed to three different doses for three days by gavage. DNA strand breaks were investigated in seven tissues (blood, bone marrow from femur, liver, spleen, kidney, duodenum, and colon) with the alkaline comet assay. Their results lead them to conclude that a short-term oral exposure to SAS NPs did not induce DNA damage in various organs of rats. However, they state that the negative systemic toxicity could be related to the low bioavailability of orally administered NPs with a short-term exposure. They open the possibility that some secondary genotoxic effects can occur after a long-term exposure.

Currently, there are plenty of projects focused on studying the toxic effect of different NPs. The caLIBRAte project[1], *which* has received funding from the European Union's Horizon 2020 Research and Innovation Programme under Grant Agreement 686239, has different work packages (WP) to fulfill different requirements. The WP 5 (*Data collation for calibration and gap analysis*) of the caLIBRAte aims to generate *in vivo* hazard data for addressing specific questions regarding NM toxicities (e.g. role of porosity) that remain unanswered.

[1] The caLIBRAte project is leaded by the National Research Centre for the Working Environment (Denmark) and the Natural Environment Research Council (United Kingdom).

1.2. Silica nanoparticles

Nanomaterials (NM) can be found in nature but they can also be synthesized to fulfill the requirements of diverse technologies and for the development of new consumer products. These novel materials (Engineered Nanomaterials, ENM) can be engineered to tune their shape, size and surface properties aiming to perform special functions. Their intended function includes catalytic activities, enhanced electrical and thermal conductivity, improved strength or controlled release of host molecules (Matsoukas, Desai and Lee, 2015).

Nanoparticles (NP) are often considered as particles with sizes ranging from 1 to 100 nm (according to the Recommendation on the European definition of a nanomaterial (2011/696/EU)) and they are classified depending on their properties: composition (at least organic or inorganic), shape or size. Depending on their applications, human exposure can occur through different routes including inhalation, dermal absorption or ingestion, and several others routes as in the case of drugs, such as intravenous injection (Dusinska *et al.*, 2015).

Properties of materials are usually measured for large aggregations of atoms or molecules (bulk material) however, when reducing dimensions up to the nanoscale, the properties are measured for little groups of atoms (e.g. a chain of 7 atoms of iron is around 1 nm length). NMs present far higher surface-to-volume ratios than similar large scale materials. Hence, the chemical and physical properties at the nanoscale are different making NMs very attractive for different applications. Surface modifications like presence of voids, pores or steps, as well as size, contribute to change the specific surface area (SSA).

Silica is a silicon oxide with chemical formula SiO_2 that can exist either in crystalline and amorphous forms. Crystalline silica is found in nature mainly as three varieties: quartz, tridymite and cristobalite. Nanosized amorphous silica (silica nanoparticles, SiNPs) has been investigated due to their biomedical and biotechnological applications. Synthetic amorphous silica (SAS) has been used in food industry as an anti-caking agent for many years (food additive E551) (Dekkers *et al.*, 2011). This additive is based on micro-sized aggregated of nano-sized primary particles (ELC, 2009). According to Dekkers *et al.* (2011), the presence of SiNPs in food industry indicates that the general population exposure is estimated to be around 9.4 mg/kg body weight/day (Dekkers *et al.*, 2011).

SiNPs can also be synthesized with controlled size, morphology and porosity. Mesoporous SiNPs (MSiNPs) are very attractive for many nanotechnological applications such as catalysis, sensing or adsorption.

1.3. Toxicity

Toxicity refers to the ability of a certain substance to cause adverse effects in living organisms. It is very often related to the term *poisonous* which refers to the morbid, noxious or deadly response caused by a certain substance. Toxicokinetics is a branch of

study of toxicology that peruses the events that occur in the body when a substance is internalized: since its absorption until the excretion. Toxicology defines where the spectrum of risk a chemical lies on and at which dose it is expected to induce damage.

There are many factors influencing the toxic effect of a certain substance: routes of entry, dose, duration and frequency of exposure. Human toxicity is usually predicted on the basis of animal studies, so that variations between different species (interspecies) and among the same specie (intraspecies) also need to be taken into consideration (UNL Environmental Health and Safety, 2002).

In conventional chemical toxicology, dose is expressed in mass units: quantity per unit mass of body weight (mg/kg bw), quantity per unit area of skin surface (mg/cm^2), quantity per unit of volume for liquids (mg/L) or volume of substance in air per unit volume of air (mg/cm^3). However, in the case of NPs, besides the mass, SSA (m^2/g) is also used to describe dose. Some studies have shown that dose-related responses are more consistent when using SSA than mass, particularly for highly insoluble materials (Simkó, Nosske and Kreyling, 2014).

1.3.1. Main toxicological hazards

Toxicological studies are conducted with two main purposes. First, to identify the highest doses at which no adverse effects are induced and second, to identify the type of effects induced by a substance. In terms of regulatory hazard classification, the following types of toxicological hazards are considered (according to Regulation (EC) No. 1907/2006, as amended by Regulation (EU) No. 2015/830): topical toxicity and systemic toxicity (see Figure 1).

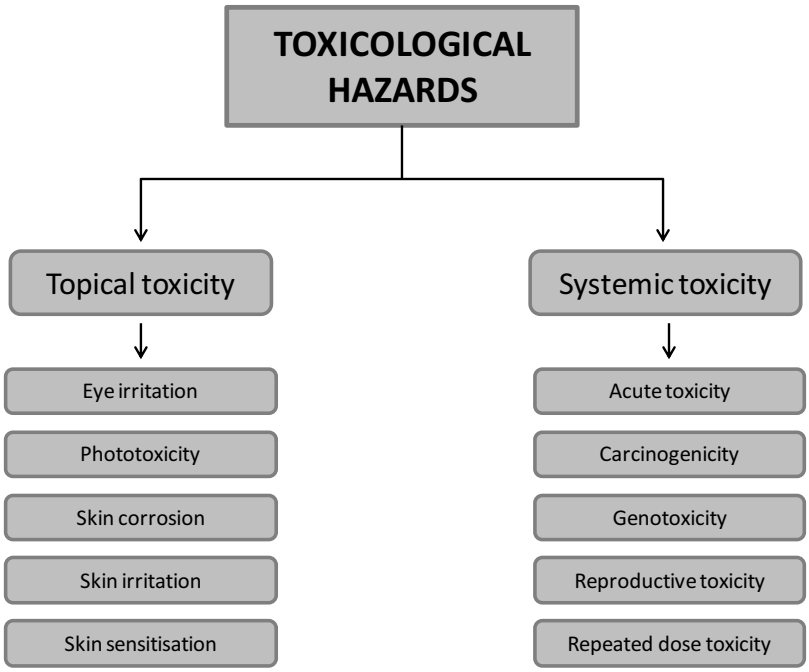


Figure 1. Classification of toxicological hazards according to Regulation (EC) No. 1907/2006, as amended by Regulation (EU) No. 2015/830.

Topical toxicity refers to all the harmful effects produced in external body organs. Eye irritation is produced when a certain substance produces changes in the eye after contact. The skin is the largest organ of the body and it provides a primary barrier for regulation and protection. Phototoxicity is the toxic response elicited after exposure to certain substances and a subsequent exposure to UV or visible light. Skin can experience harmful effects such as corrosion, irritation and sensitisation. The difference among these hazardous effects is that corrosive substances produce irreversible damage in the skin whereas irritant substances lead to reversible local inflammatory reaction caused by the innate immune system. On the other hand, sensitisation is caused by substances that are able to generate an allergic response.

Systemic toxicity is produced once the toxicant is absorbed and distributed into the body. Therefore, the substance can target different organs and trigger harmful effects at diverse levels. Acute toxicity refers to the toxicological effects that occur after a single or multiple administrations that have been performed in a short time period (usually within 24 hours). Carcinogenicity is related to hazards that lead to cancer inductions or increases in its incidence (benign and malignant tumors). Reproductive toxicity has repercussions on sexual function and fertility as well as adverse effects on development of the offspring. Genotoxicity refers to affectations related to changes in the amount or structure of genetic material involving mutations (mutagenicity) or not.

1.3.1.1. Genotoxicity

Genotoxicity defines the destructive effect of certain substances and or radiations on cellular genetic material. The substances that produce DNA or chromosomal damage are genotoxins. Because DNA is the repository of genetic information in each living cell, its integrity and stability are essential to life. However, it is subjected to the assault from genotoxins.

The results of the DNA damage depend on the cell type. If the damage happens in a germ cell, it will have the potential to be inherited (germline mutation). On the other hand, if the damage is experienced by a somatic cell and results in a somatic mutation, it can lead to a malignant transformation (cancer).

The most used assays to test the genotoxic effects of a certain substance *in vivo* are the comet assay, the micronucleus assay and the chromosome aberrations. The three assays have their own OECD guideline for the testing of chemicals (TG 489, TG 487 and TG 473 respectively).

- Alkaline Comet assay

  The comet assay or Single Cell Gel Electrophoresis (SCGE) is a method used to detect and quantify DNA damage in individual eukaryotic and prokaryotic cells and therefore, it is used to identify genotoxic substances. In 1984 Ostling and Johanson (1984) proposed a microelectrophoretic study to detect DNA damage consisting in an electrophoresis of cells immersed in agarose. Subsequently, Singh and colleagues (1988)modified the assay in 1988 changing the neutral conditions into alkaline

conditions for detection of single-strand breaks (SSB), double-strand breaks (DSB) and alkali labile sites. Since alkaline comet assay was proposed, it has been subjected to many different modifications and improvements for detection of oxidative base damage or DNA cross-linking with DNA. Nevertheless, the assay mainly consists in cell isolation, cellular lysis, DNA unwinding, electrophoresis and staining.

Comet assay is a versatile and sensitive compared to other genotoxicity tests such as chromosomal aberrations, sister chromatid exchanges, alkaline elution or micronucleus assay. Comet assay is capable for detecting low levels of DNA damage, which is a requirement for small number of cells per sample. Moreover, it has an ease application, is low costing and is relatively easy to perform. However, the assay also presents some drawbacks. It is not able to detect strand breaks that have been repaired, it can show technical and interpretation variability (Dhawan Alok; Anderson Diana, 2017).

The assay consists in different consecutive steps. First, cells must be isolated and embedded in agarose. Then, agarose drops are placed onto a film slide (support) and after their solidification, the slides are subjected to the following steps.

As the subject of study is DNA, the procedure is started with the lysis to break cell membranes and free DNA. The films are submerged in lysis solution consisting of detergent and high salt concentration. Once the cells are lysed, DNA loops remain attached to the matrix, for that reason films are submerged in alkaline solution (pH>13) for DNA unwinding. The alkaline solution allows DNA to unwind and release relaxed DNA loops and fragments.

The DNA in the agar is then subjected to electrophoresis also run under alkaline conditions. In this step two different scenarios occur depending on the DNA conditions: normal non-fragmented DNA molecules remain in the position where the nuclear DNA had been in the agar, while any fragmented and relaxed DNA loops migrate towards the anode leaving the tail and generating the comet shape. The extent of DNA that has migrated during electrophoresis and the migration distance reflects the amount and size of DNA fragments. The level of DNA migration is linearly associated with the duration of the electrophoresis, and also with the potential. Therefore, it is crucial to keep constant the same electrophoresis conditions to be consistent from run to run.

After completion of the electrophoresis, the unwinding is neutralized submerging the films in PBS and miliQ water. The last step consists on the fixation of cells with pure ethanol.

In order to perform the comet scoring, cells are stained with a nucleic acid stain and checked with fluorescent microscopy. Compared with the homogeneous staining of neutral comets, the DNA in the tail of an alkaline comet appears granular, as if DNA fragments are present (Collins *et al.*, 2008) (OECD TG 489, 2014).

Additionally, to increase the specificity of the assay, there are some treatments that can be included to the protocol in order to detect the type of DNA damage. FPG-modified comet assay (formamidopyrimidine DNA-glycosylase) includes a step for digesting the nucleoids with the enzyme that recognizes specific oxidative DNA lesions including 8-hydroxy-2-deoxyguanosine (8-OHdG) and creates a break (Gerloff *et al.*, 2009).

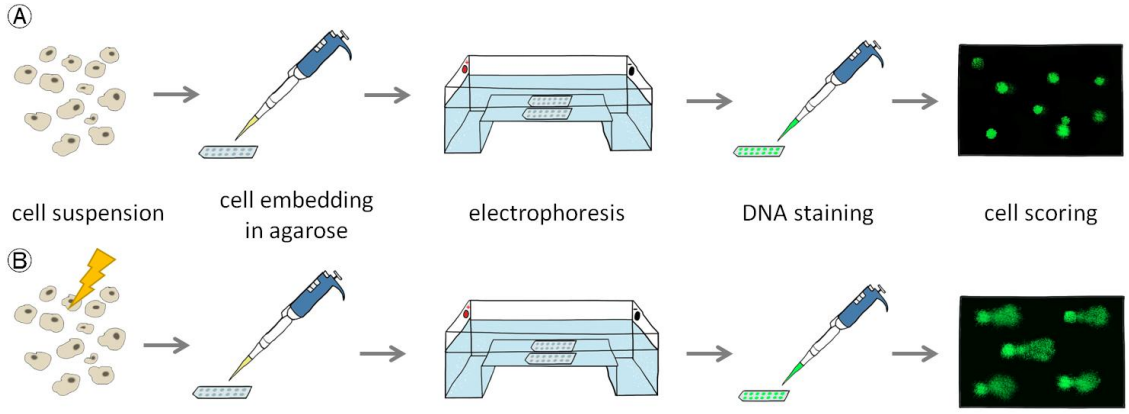


Figure 2. Comet assay performance (simplified). (A) Undamaged cells are observed as dots after electrophoresis while (B) damaged cells present tails and are observed as comets.

- Micronucleus assay

The micronucleus assay (MN) is a method used for screening substances that produce chromosome breakage or loss effects. Micronucleus were recognized in the end of the 19th century when Howell and Jolly found small inclusions in the blood taken from cats and rats, they named them Howell Jolly bodies. These inclusions are also observed in the erythrocytes.

MN assay is proven to be an effective measure of the genotoxic potential of a certain substance. However, the sampling is performed evaluating just a single tissue (bone marrow) as an indicator of genetic in vivo damage. Therefore, the assay provides a limited genotoxicity profile.

Bone marrow is the target tissue for genetic damage in this assay due to its high vascularization and since erythrocytes are produced in this tissue. The utilization of peripheral blood for the measurement of micronuclei is only acceptable in case for substances that cause structural or numerical chromosomal aberrations in these cells. The frequency of micronucleated erythrocytes is the principal endpoint. For assays performed with bone marrow, immature erythrocytes are used. On the other hand, for assays performed with peripheral blood, mature erythrocytes are used.

As erythrocytes are the subject of study, the procedure is started by extracting the bone marrow or collecting blood. Then, the preparations are made and stained. Finally, preparations are analysed for the presence of micronuclei either by visualization using a microscope, image analysis, flow cytometry or laser scanning cytometry (OECD TG 487, 2014).

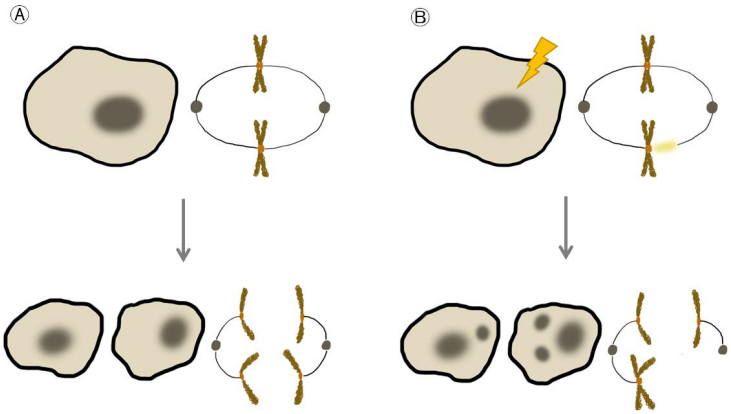


Figure 3. Micronucleus assay (simplified). (A) Undamaged cells generate normal daughter cells after mitosis, while (B) damaged cells present altered cells with multiple nuclei after mitosis.

- Chromosome Aberration assay

The chromosome aberration assay is used to detect structural chromosome aberrations induced by a certain substance in bone marrow cells. Structural chromosome aberrations may be of two types, chromosome-type if they involve both chromatids, or chromatid-type if they only involve one of them. The most common aberration type is the chromatid-type. However, chromosome-type aberrations also occur and they are often related to alterations in oncogens and tumor suppressor genes. An increase in polyploidy (4n) gives information that a chemical has the potential to induce numerical aberrations.

Bone marrow is also the target tissue in this test, since it is a highly vascularised tissue, and it contains a population of rapidly cycling cells that can be readily isolated and processed.

Once bone marrow is obtained, it is exposed to hypotonic solution and fixed. The cells are then spread on slides and stained (normally with Geimsa stain). The mitotic index should be determined as a measure of cytotoxicity by visualization of chromosomes during metaphase (OECD TG 473, 1997).

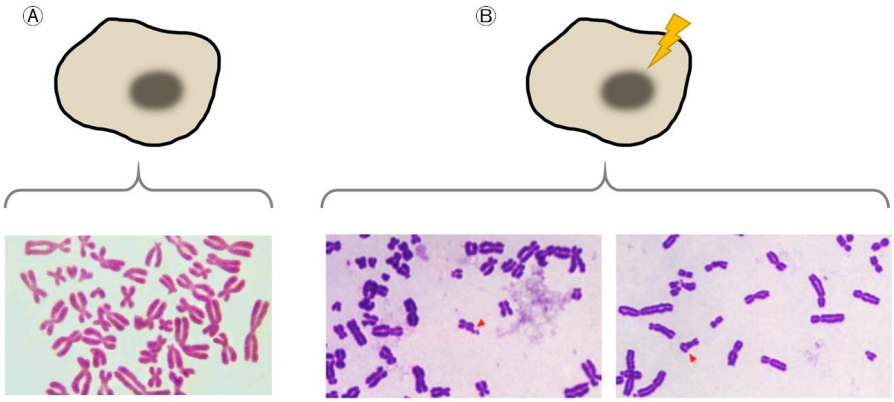


Figure 4. Chromosome aberration assay performance (simplified). (A) Undamaged cells chromosomes do not present alterations, while (B) damaged cells chromosomes present structural defects.

1.4. Nanotoxicity

Nanomaterials (NM) are being applied in many fields of science like physics, chemistry, pharmaceutical science, material science, medicine and agriculture among others. NMs are characterized mainly by their size, which belongs to the border between individual atoms or molecules and the corresponding bulk materials. Physicochemical characteristics of NPs such as size, shape, surface properties (i.e. SSA), crystal state, aggregation/agglomeration state, etc. impact in the activity that they perform and can contribute to generate harmful effects in different organisms. Nevertheless, the relation between these properties and toxicity is still not well understood (Colvin, 2003). An increase in SSA implies a larger number of free radicals and ions arising from NP dilution, and therefore, it also implies an increase of their reactivity (Magdolenova *et al.*, 2014).

1.1.4. Toxicokinetics of NMs

When external exposure to NMs occurs, these may be absorbed. Absorption and subsequent toxicokinetics processes are described below.

- Absorption of NMs

  Absorption is the process in which the substance (NM) enters the body. It is mainly produced by three different routes: through inhalation, orally or via dermal contact. Inhalated primary NPs and small aggregates/agglomerates are removed by macrophages when they reach the alveoli. Orally absorbed NPs NM are mostly excreted with the feces when they reach the gastrointestinal tract (GI). Nevertehless, low levels of NPs are absorbed. The absorption of NPs by the GI tract depends on the paricle surface charge; positively charged NPs are more efficiently internalized compared to their negative and neutral counterparts. Most of the studies performed about dermal absorption of NPs have shown that unintentional permeation of the particle through the skin is not observed. Permeation occurs after physical damage to the skin (Landsiedel *et al.*, 2012).

  According to Oberdörster *et al.* (2005) in the field of nanotoxicology research, inhalation and ingestion are considered as two major uptake routes of NPs to the human body and once they are inside, they can enter into the cells via active or passive uptake mechanisms. The ability to generate a toxic response of some ENPs in the gastrointestinal tract is still largely unknown. While Lomer *et al.* (Lomer *et al.*, 2004) estimated that around 10^{12}–10^{14} microparticles are already ingested daily per person, the food industry expects to increase dramatically the application of ENPs in the near future. For that reason, the research to increase knowledge on the potential toxicological effects of orally internalized NPs is crucial.

- Distribution of NMs

  After the absorption process, NPs get into the blood or lymphatic system to be distributed through the body. This mechanism is highly dependent on the capillary blood flow to the tissues and the ability of the substance to pass through cell membranes of a certain tissue. The distribution of NPs through a certain route is dependent on their size and surface modifications. The major uptake of NP is in macrophage rich organs like liver, spleen, lymph node and bone marrow.

- Metabolism of NMs

  In contrast to most xenobiotics, NPs are found predominantly not metabolized. However, there are some exceptions for functionalized NPs (e.g. NPs with biological molecules attached in their surface).

- Excretion of NMs and recognition by the Immune System

  Finally, the body has its own mechanism to eliminate and expulse NPs that have not been stored. When NPs are in the blood stream, they can be recognized by the Mononuclear Phagocyte System (MPS) or by macrophages in some organs. The recognition by the Immune System is dependent on the NP surface charge. Kidneys are the most important organs for removal of toxins from the body by excretion via urine (urinary excretion) or via feces through the intestinal tract (hepatic and fecal excretion). However, some NPs are biopersistent and remain in organs for several months.

Taking into account that this report is based on evaluating the genotoxic effects of SiNPs *in vivo*, a short overview of the toxicity of SiNPs is provided below.

Crystalline SiNPs are considered carcinogenic (Group 1 as evaluated by IARC[2]) but amorphous SiNPs are not (Group 3 as evaluated by IARC[3]).

A literature review about toxicology of amorphous SiNPs (Murugadoss *et al.*, 2017) presented results from several *in vivo* toxicity studies carried out using rats and mice and exposure through various routes. From the review the following information can be extracted. Intravenous injection caused toxicity in all the studies. In general, most of the SiNPs administered orally and inhaled to rodents were excreted via feces and urine (Dekkers *et al.*, 2011). SiNPs with modified surfaces showed a significant increase in absorption by the gastro intestinal tract compared to bare SiNPs. In general, short-term exposure (acute studies) produced toxic effects in kidneys, liver, brain and lungs. Chronic oral studies with amorphous SiNPs showed adverse effects in liver at very high doses (1500 mg/kg bw). Nevertheless, in most circumstances, the administered doses were very high compared to relevant human inhalation and ingestion exposures to amorphous silica.

[2,3] International Agency for Research on Cancer (IARC) Group 1 substances are considered carcinogenic to humans. Group 3 substances are not classifiable as to its carcinogenicity to humans.

1.4.1. Genotoxic effects of NPs

The mechanisms of nanoparticle genotoxicity are still not well understood and it is often not clear if an effect on DNA is nano-specific. NPs can produce genotoxic effects once they are internalized into the body and absorbed into a target cell. Equally to the mechanisms of regular toxicity, in the specific case of genotoxicity, the effects generated by NPs can be either caused directly or indirectly (see Figure 5).

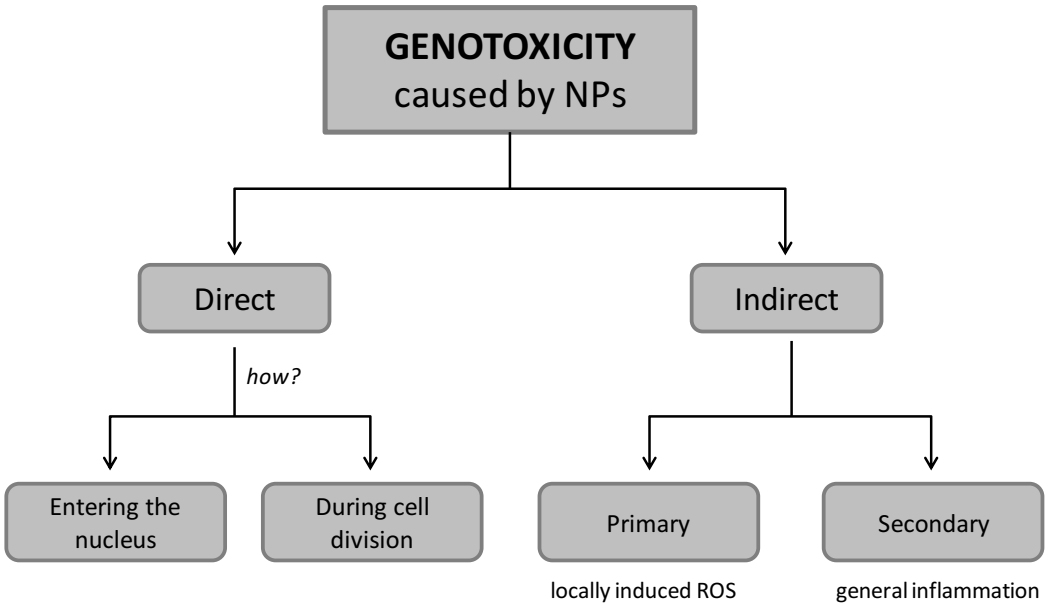


Figure 5. Different causes of genotoxicity produced by NPs.

Direct genotoxic effects caused by NPs depend on the cell cycle and their targets are described for interphase and mitosis. Direct genotoxicity is produced by mechanical interference of NPs with cellular components. Due to their size, NPs are able to interact and interfere with cell components with dimensions at the same size range; nucleosomes (11 nm) only if the NP is able to enter the nucleus, microtubules (25 nm), actin filaments (7 nm) or centrosomes (25 nm) (Gonzalez, Lison and Kirsch-Volders, 2008). As these components play an important role in cell division, their interference can produce cell division dysfunctions.

Base modifications and DNA single strand breaks (SSB) can be induced during all the stages of the cellular cycle through indirect genotoxicity. Increased levels of ROS can be generated by particles either through particle-cell contact (primary indirect genotoxicity) or as a consequence of an inflammatory response generated by leukocyte detection of NPs (secondary indirect genotoxicity). Moreover, there are other possible manifestations of genotoxicity related to NPs as metal release from metallic soluble NPs or desorption of organic components among others. However, these genotoxic effects appear less specific to NPs (Gonzalez, Lison and Kirsch-Volders, 2008).

The testing of the genotoxic effects caused by NPs can be performed *in vitro* or *in vivo*. The *in vitro* assays are useful for testing primary genotoxicity whereas *in vivo* assays also

give information about secondary effects such as inflammation (Magdolenova *et al.*, 2014).

Inside the cells, NPs are able to generate reactive oxygen species (ROS) like superoxide ($O_2^{\bullet -}$), hydroxyl radicals ($^{\bullet}OH$) or hydrogen peroxide (H_2O_2) as well as reactive nitrogen species (RNS) like nitric oxide (NO) and peroxynitrite ($ONOO^-$) in a direct or indirect manner (Unfried *et al.*, 2007) (Gerloff *et al.*, 2009). Formation of ROS can occur directly or indirectly. The chemistry of particles can lead to direct ROS formation, whilst some cellular targets can be activated to produce it in an indirect way (Brown *et al.*, 2004). Moreover, particles can lead to indirect ROS generation by inducing inflammation characterized by the recruitment and activation of ROS producing phagocytic cells. Particles able to induce oxidative stress by generating ROS have been proposed as toxic candidates. Such properties have been associated not only with the activation of inflammatory mediators, but also with the induction of oxidative DNA damage and associated mutagenesis (Schins and Knaapen, 2007) (Gerloff *et al.*, 2009).

According to the literature review about nanomaterials and genotoxicity performed by the Swedish Chemicals Agency (KEMI, 2016), the *in vivo* studies on genotoxicity of amorphous SiNPs conclude that no effects are triggered after inhalation (rats following 1 or 3 days inhalation evaluated with MN assay) either intratracheal exposure (evaluated with comet assay). The studies using oral exposure to SiNPs showed a weak effect in colon of rats (evaluated with MN assay) but no induction of DNA damage in other tissues (evaluated with comet and MN assays). Finally, for the studies that used intravenous injections as route of exposure, the results showed no signals of DNA damage in various tissues (evaluated with MN assay) except for liver and blood cells.

2. AIM OF THE PROJECT

The main objective of this project is to evaluate if SiNPs administered by the oral route in mice induce genotoxic effects, and to investigate a potential role of porosity on such effects. This study is part of Work Package 5 (*Data collation for calibration and gap analysis*) of the caLIBRAte European H2020 project. One of the aims of this WP consists on generating *in vivo* hazard data for addressing specific questions regarding NM toxicities (e.g. role of porosity) that remain unanswered.

2.1. Validation of the *in vivo* assay setup

The comet assay consists in several different long-lasting steps and, if it is performed in one go, it can last the whole workday. Indeed, the protocol was established in the laboratory for *in vitro* assays. When it is conducted on tissues derived from an *in vivo* study, the duration of the comet assay needs to be added to the time necessary to prepare the specimens (animal sacrifice, tissue harvesting, cell disaggregation and preparation of the desired cell dilution), resulting on timings that are rather impractical. Therefore, one of the goals of the project was to adapt the *in vitro* assay setup to the whole *in vivo* experiment setup.

2.2. Evaluation of the genotoxicity of SiNPs with comet assay

This project is focused in evaluating the genotoxicity of SiNPs *in vivo* with the alkaline comet assay after a short-term oral administration and it is framed in a broader project with different toxicology-related endpoints.

The setup of the assay aims to determine if SiNPs are genotoxic and to evaluate the influence of their porosity (directly related to SSA) with potential genotoxic effects.

3. MATERIALS AND METHODS

3.1. Materials

The following tables contain all the materials that have been used for all the experiments.

REAGENTS		
Reagent or compound	**Manufacturer**	**Reference**
DMEM	Cultek	BE12-614F
DMSO	Sigma	D-8779
EDTA	Sigma	E9884
EMS	Alfa Aesar	A12938
Ethanol	Merck	1,00983,1000
FBS	Cultek	SH30070.02
HBSS	Invitrogen-Gibco	14065-049
Low gelling Point Agarose	Sigma	A9414
MiliQ water	-	-
MMS	Sigma	129925
NaOH (pellets)	Panreac	131687
PBS	Sigma	D8537
Pen-Strep	Lonza	H3DE17-602F
Sodium Chloride	Panreac	131659
Sodium Salt CMC	Sigma	C5013
SYBR Green	Sigma	S9430
Transferrin	Sigma	T8158
Tris (Trizima hydrochloride)	Sigma	T1503
Triton	Sigma	T8532
Trypan Blue	Invitrogen	T10282
Trypsin-EDTA	Gibco	25200-072
Tween80	Sigma	P1754

ANIMALS	
Specie	Mice
Strain	SWISS (RjOrl:SWISS_SPF)
Source	Janvier-Labs (France)
Age and Sex	4 weeks old female
Body weight at arrival	20-24 g
Food	Protein rodent maintenance diet from Harlan

CELLS	
Cell line	Caco-2
Clone	C2BBe1
Source	ATCC

NANOMATERIALS				
SiNP diameter	**Porosity?**	**ID (from NRCWE)**	**SSA after methyl coating (m^2/g)**	**Manufacturer**
300nm	Porous	NRCWE#065	828	National Research Center for the Working Environment (NRCWE), Denmark
	Non-porous	NRCWE#066	10	
100nm	Porous	NRCWE#067	844	
	Non-porous	NRCWE#068	22	

EQUIPMENT	
Equipment	**Manufacturer**
Cell counter	Invitrogen Countess
Centrifuge	Gyrozen 1248R
Electrophoresis Tank	-
Epifluorescence Microscope E600	Nikon
Digital camera (Epifluorescence Microscope)	Olympus DP72 color
High Resolution TEM	JEOL Ltd
Digital camera (HRTEM)	Megaview III CCD
pH-electrode	Hach
pH-meter	Crison GLP21
Power Supply	Power Pac HC
Sonication Probe	Sigma Tappered Microtip
Thermoblock	P Slecta
750W Sonicator	SONICS Vibracell USA

CONSUMABLES	
Material	**Manufacturer**
Cell strainer	Corning
Counting slides	Nano Entek
Gelbond® Slides	Lonza
Glass slide	SuperFrost Plus VWR
T75 Flask	Falcon
6-well plate	Costar

SOFTWARES	
Software	**Purpose**
Comet Assay IV	Comet scoring
Cell ^F	Comet Image adquisition
Graphpad Prism	Statistical analysis
AnalySIS (SIS, Munster)	TEM image adquisition

3.2. Methods

The whole consortium participating in the caLIBRAte project takes into account and follows the European Commission Recommendation of 07/02/2008 on a Code of Conduct for a Responsible Nanosciences and Nanotechnologies Research(European Union, 2009). In addition, all the experimental procedures with live animals were already approved by the ethical committee of PCB and the local government (Generalitat de Catalunya) in accordance with the European Communities Council Directive 2010/63/EU and were performed by certified personnel in the necropsy facilities from the stable at Parc Científic de Barcelona (PCB).

3.2.1. Alkaline comet assay

The method for performing the *in vitro* and *in vivo* alkaline comet assay has followed the principles described in the protocol from previous studies at the laboratory, which was based on the OECD TG 489 guideline for the testing of chemicals *in vivo* (OECD TG 489, 2014).

This guideline includes the recommended use and limitations of the comet assay. It is based on the final protocol used in the validation trial, and on additional relevant published and unpublished (laboratories proprietary) data.

The *in vivo* comet assay that was conducted in this project was part of a bigger experiment that included additional toxicological endpoints. To fit the comet assay within the logistics of the whole experiment, some adaptations where included to basically allow the performance of the protocol within two working days. Hereunder, the general steps that were followed to perform an *in vitro* or an *in vivo* comet assay are described.

1. Preparation of Specimens (cell suspension)

The only step in which the *in vitro* and the *in vivo* comet assay differ is the preparation of cell suspensions. Cell samples were prepared immediately before starting the assay. Cell samples were handled under dimmed or yellow light to prevent DNA damage from ultraviolet light. Buffers were cooled to 4°C to inhibit endogenous damage occurring during sample preparation and to inhibit repair in cells. PBS was Ca^{+2} and Mg^{+2} free to inhibit endonuclease activities.

1.1. *Cell from culture (adherent cells)*

Cells were gently detached from flask surface using Trypsine-EDTA. Cells and medium were transferred to a vial, centrifuged (5 min, 1000 rpm, 22ºC) and the supernatant was removed. Cells were resuspended in 2 mL of ice-cold PBS and cell counting was performed. Finally, the cell suspension volume was determined to have $2{,}5x10^5$ cells/mL in a final volume of 1 mL.

1.2. *Cell disaggregation from tissue*

A piece of 6 cm of intestine (jejunum) was harvested from the animal and placed into 3-4 mL of ice-cold Mincing Buffer (1X HBSS Ca^{+2} and Mg^{+2} free, 20 mM EDTA and 10% DMSO (v/v), pH=7,5).

First, the tissue was cleaned removing blood capillaries, mucous membranes or intestinal content. Then, it was opened and the ends from which the piece was manipulated were discarded (1 cm from each end). Finally, using small dissecting scissors the tissue was minced into very small pieces (1 – 2 mm^3) and suspended in ice cold PBS.

Later, the cell suspension was recovered with pipette avoiding transfer of debris and passed through a cell strainer (100 µm pore size; Corning REF: 431752 or similar) using a syringe plunger. The vial containing the cell suspension was centrifuged (5 min, 1000 rpm, 22ºC) twice. After discarding the supernanat, the pellet of cells was resuspended in 2 mL of PBS and is proceeded to count cells. Thereafter, PBS is added to obtain a concentration of $2,5x10^5$ cells/mL, which is the desired concentration of cells for embedding in agarose.

2. Embedding cells in agarose and preparation of GelBond® films

Using 30µL of the $2,5x10^5$ cells/mL cell suspension (7500 cells) combined with 270µL of Low Melting Point Agarose (0,75% LMPA, PBS 1X Ca^{+2} and Mg^{+2} free, EDTA 10mM, pH 7,4) previously melted (a peak of 80ºC temperature was applied in a Thermoblock and then kept at 40ºC) provided a final agarose concentration of 0,65% and a cell density of 175 cells per drop when spreading 7µL drops in a GelBond® film.

To dispense the drops, the GelBond® film was placed on top of a pre-cooled alumina plate with a cooler block ice placed below. Six drops of 7 µL per condition were distributed on the film. Once all the drops were settled, the GelBond® film was cooled down (5 min, 4ºC) to ensure the solidification of LMPA.

3. Lysis

After solidification of agarose, the slides were immersed in lysis solution (NaCl 2,5 M, EDTA 0,1 M, Tris 10 mM, Triton 1% (v/v) and DMSO 10% (v/v), the later only for samples containing heme, such as blood cells or tissue samples, pH=10) and kept overnight at 4ºC and at the darkness.

Note: The decision of leaving the slides in lysis overnight is the first adaptation of the assay. The OECD guideline suggests a minimum time of 1 hour of lysis but longer times can also be applied.

4. DNA Unwinding and Electrophoresis with alkaline treatment

Before proceeding with the electrophoresis, the GelBond® slides were washed during 5 minutes with alkaline solution (NaOH 0,3 M, EDTA 0,001 M, pH>13, 4ºC) and then left 1 hour covered by the solution at 4ºC at the darkness to unwind the DNA.

The slides were placed onto the platform ensuring a correct balance design and glued to the tank with scotch tape to avoid migration. The submarine-type electrophoresis tank was filled with sufficient electrophoresis solution (same composition as alkaline solution) to cover completely the slides. The electrophoresis conditions were: potential of 0,7 V/cm, current of 300 mA, duration of 20 minutes at 4ºC. The current was set by adding or removing electrophoresis buffer from the tank.

5. Neutralisation

After completion of electrophoresis, the slides were washed with 1X PBS during 5 minutes twice and once with MiliQ water during 1 minute at 4ºC for neutralizing the effect of the unwinding solution.

6. Fixation and drying

The slides were rinsed with Ethanol (100% (v/v)) once before incubating overnight. Finally, the slides were removed from the ethanol and placed in a container at the dark to let them air dry at least 2 hours at room temperature.

Note: The decision of leaving the slides fixing overnight is the second adaptation of the assay. The OECD guideline suggests a minimum time of 5 minutes in Ethanol but longer times can also be applied.

7. Staining

Once the agarose was dry and fixed to the slide, the DNA was stained with SYBR Green solution (1:100 diluted in a buffer of Tris 0,01 M and EDTA 0,001 M at pH=8) during 3 minutes making sure the gels were covered with the staining solution at complete darkness. After the completion of these minutes, the slides were rinsed with distilled water and let dry.

8. Visualization and Scoring of comets

For visualization of the cells, the epi-fluorescence microscope (E600 Epifluorescence Microscope, Nikon, Japan) and an appropriate digital camera were used. The slides were mounted in a microscope slide and cover slips *in situ* for viewing.

A FITC filter (460-500nm excitation, 510-560nm emission) was used in the microscope. The maximum excitation wavelength of SYBR Green is 497nm, but there is also a secondary excitation peak near 254nm. The fluorescence emission of SYBR Green stained DNA is centered at 520nm.

Images were taken at 10 X magnification, ISO 200 live/snap 800 ms and with monochrome filter. The image capturing was performed using the Cell^F Software (3.2 (Build 1700), Olympus Soft Imaging solutions GmbH, Japan).

For the cell scoring, at least 100 nuclei were scored per animal using Comet Assay IV Software (version 4.2, Perceptive Instruments Ltd, UK).

Overlapped cells (Figure 6A) or cells close to the edge of the picture (Figure 6B) are not scored to avoid incorrect measurements.

Figure 6. Examples of avoided cells for scoring (A) overlapping cells (B) cells at the edge.

Cells suitable for scoring are normally identified by the scientist and the software semi-automatically calculates different parameters. These include head and tail length, head and tail intensity, tail moment and total area. The most commonly used parameter for statistical analysis is tail intensity (also known as % tail DNA). Unlike other endpoints, tail intensity can be compared across studies and laboratories, and also is linearly related to DNA damage. Tail intensity values can range from 0% (no DNA damage) to 100%. Cells presenting % tail DNA above 80% correspond to cells with very small or non-existing heads (Figure 8). These cells are called "hedgehogs" and their origin is still under discussion but it is thought that they represent dead or dying cells (Hobbs *et al.*, 2015). Following the recommendations of the OECD guidline, "hedgehogs" are excluded from scoring.

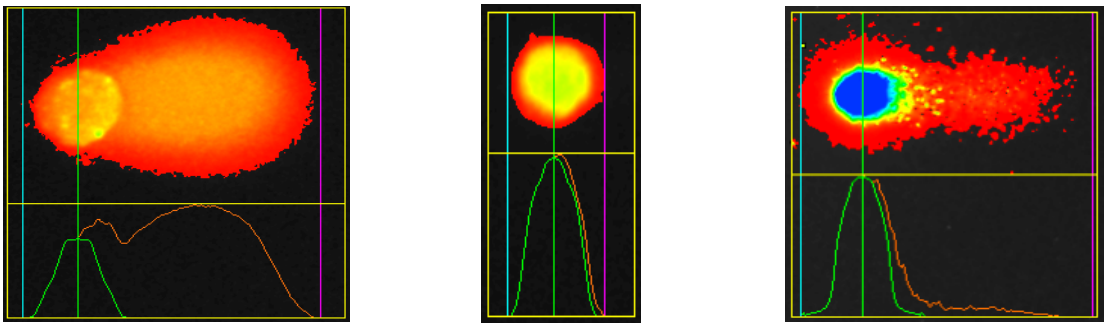


Figure 7. Different types of cells after comet assay imaging.

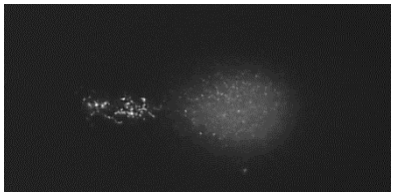


Figure 8. Cell considered as "hedgehog".

After scoring around 100 cells, the median of all the tail intensity values was calculated. The mean of medians for the 6 animals included in each experimental condition, as well as the SEM was then calculated.

9. Storage and re-visualization

To store the slides, the cover slips were removed. To do it, the slide was immersed in miliQ water until the cover slip was detached. Once the sample was removed from the slip, it was stored at room temperature conditions and for a re-visualization; the staining process was repeated.

3.2.2. Validation of the *in vivo* comet assay setup

The comet assay had been used in several previous studies at the laboratory. However, the protocol that was followed had to be changed as already mentioned to facilitate the implantation into the *in vivo* study. Such modifications might affect the sensitivity of the test. Therefore, prior to the main experiment of this project, series of preliminary studies were performed to ensure that the protocol for this experiment had no repercussion on the sensitivity of the assay.

3.2.2.1. Implementation in vitro for validation of selected assay conditions

To check if the implementation of the two selected adaptations produced acceptable results (i.e., produced reproducible results and were able to identify damage produced by a positive control), the protocol was first conducted *in vitro* with human intestinal cells. The assay was conducted with two genotoxic positive controls as a validation tool of the test.

Methyl Methane Sulfonate (MMS) and Ethyl Methane Sulfonate (EMS) were selected, as they have been reported to produce DNA strand breaks in the intestine as well as *in vitro* (see Table1) (Tarantini *et al.*, 2015) (OECD TG 489, 2014).

Table 1. Types of DNA lesion and major mutations type produced by EMS and MMS (OECD TG 489, 2014).

	TYPE OF DNA LESION	MAJOR MUTATION TYPE
EMS	Alkylation	Base-pair substitution
MMS	Alkylation and Strand breaks	Several different types

For the *in vitro* comet assays, human colon epithelial cells (C2BBe1 (CACO-2 subclone) from the ATCC) were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 1% Penicillin / Streptomycin (Pen/Strep) and 200 µL of Transferrin. Cells were incubated at 37°C under 5% CO_2 and water saturated atmosphere. For maintenance, cells were cultured in flasks and sub-cultured once a week when they reached around 80% confluence. To conduct the comet assay, cells were seed in 6 well plates at a $4x10^5$ cells per well and grown for 24 h before exposure of control compounds.

Three conditions (two replicates per condition) were tested: ① cells exposed to control cell culture media, ② cells exposed to MMS at 40 µg/mL and ③ cells exposed to EMS 200 µg/mL. Cells were exposed to 2 mL of each of these substances during 24 hours. Afterwards, they were trypsinized and centrifugated (5 min, 1000 rpm, 22ºC).

The pellet obtained was resuspended in a small volume and the cell concentration was evaluated using a cell counter. Then, PBS was added to obtain a cell concentration of $2,5x10^5$ cells/mL and a final volume of 0,5 mL.

The cell suspension (30 µL) was mixed with 270 µL of low melting point agarose (LMPA) at 37ºC. Drops of 7 µL of the embedded cells were placed onto the GelBond® film in an ordered pattern (Figure 9). Then, the film was put 5 minutes at 4ºC to condense the agarose. After that, the lysis, alkaline treatment, electrophoresis, neutralization, fixation and evaluation (staining, visualization and scoring) steps were carried out following the parameters depicted in section 3.2.1.

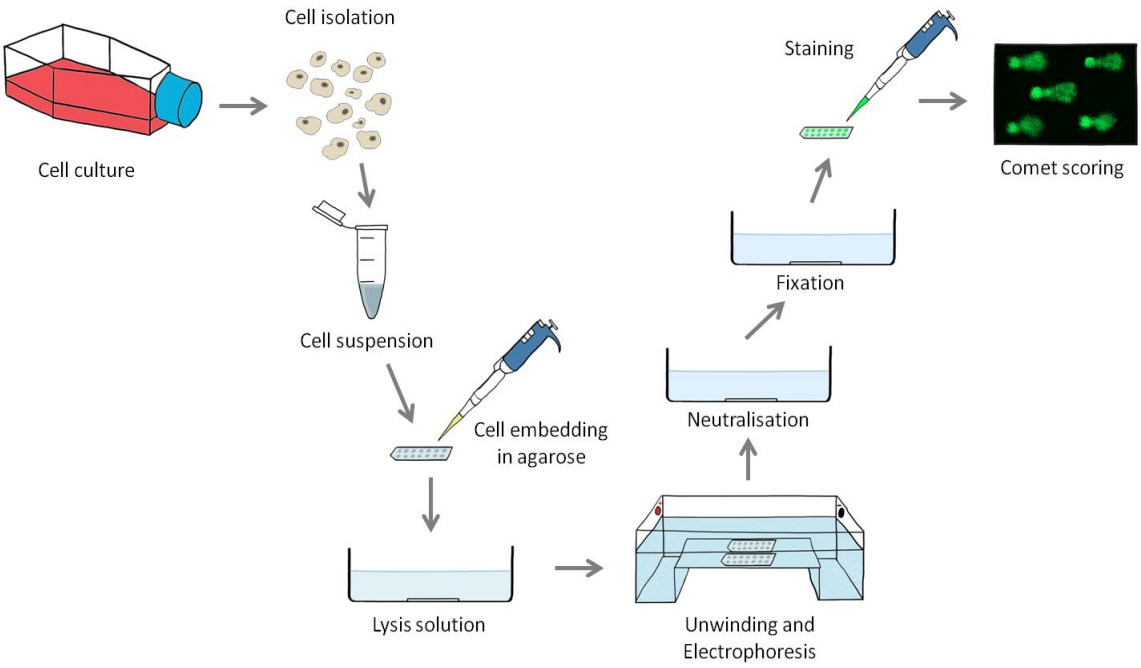


Figure 9. Steps involved in the performance of the alkaline comet assay *in vitro*.

3.2.2.2. Implementation in vivo

- Validating the modified comet assay setup

Once the adapted protocol was proved to present sensible results *in vitro*, it was applied to cells obtained from fresh mice intestinal samples. The assay setup was initially performed using intestine (jejunum) samples (Figure 10) from four weeks old C57BL/6 female mice (25-30 g body weight from Janvier Labs, France). Three sets of experiments including 3 mice per experiment were performed.

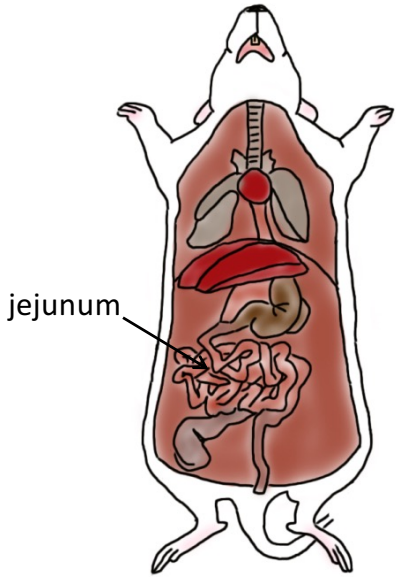


Figure 10. Anatomy of the mouse.

Mice received two administrations of 100 mg/kg MMS (positive control) or with vehicle (saline solution, 9% NaCl, vehicle control). These oral gavages administered were performed 24 hours and 3 hours before sacrifice.

Mice were sacrificed using CO_2 and samples from intestine (6 cm of jejunum) were harvested and kept in ice-cold mincing buffer (1X HBSS Ca^{2+} and Mg^{2+} free, 20 mM EDTA and 10% DMSO (v/v), pH=7,5).

Tissues were then cleaned by removing blood capillaries, mucous membranes or intestinal content. Then, the intestine was opened and the ends from which the piece was manipulated were discarded (1cm from each end). Finally, using small dissecting scissors the tissue was minced into very small pieces (0,5 – 1 mm^3) and suspended in ice-cold PBS (1X).

The tissue small pieces were transferred to a 100 µm cell strainer coupled to a test tube and there they were softly smashed to make them pass through the strainer. Finally, the tissue cellular content was suspended in 10 mL of PBS. The sample was centrifuged (1000 rpm, 5 min, 23ºC) and washed twice. Later, the pellet was resuspended in 2 mL of

PBS and the cell counting was performed to determine the volume of PBS needed for obtaining the concentration of $2{,}5x10^5$ cells/mL and a final volume of 1 mL.

The remaining steps concerning the alkaline comet assay (lysis, unwinding, electrophoresis, neutralization, fixation and evaluation) were performed as previously explained in the methodology section 3.2.1 following the final adopted protocol.

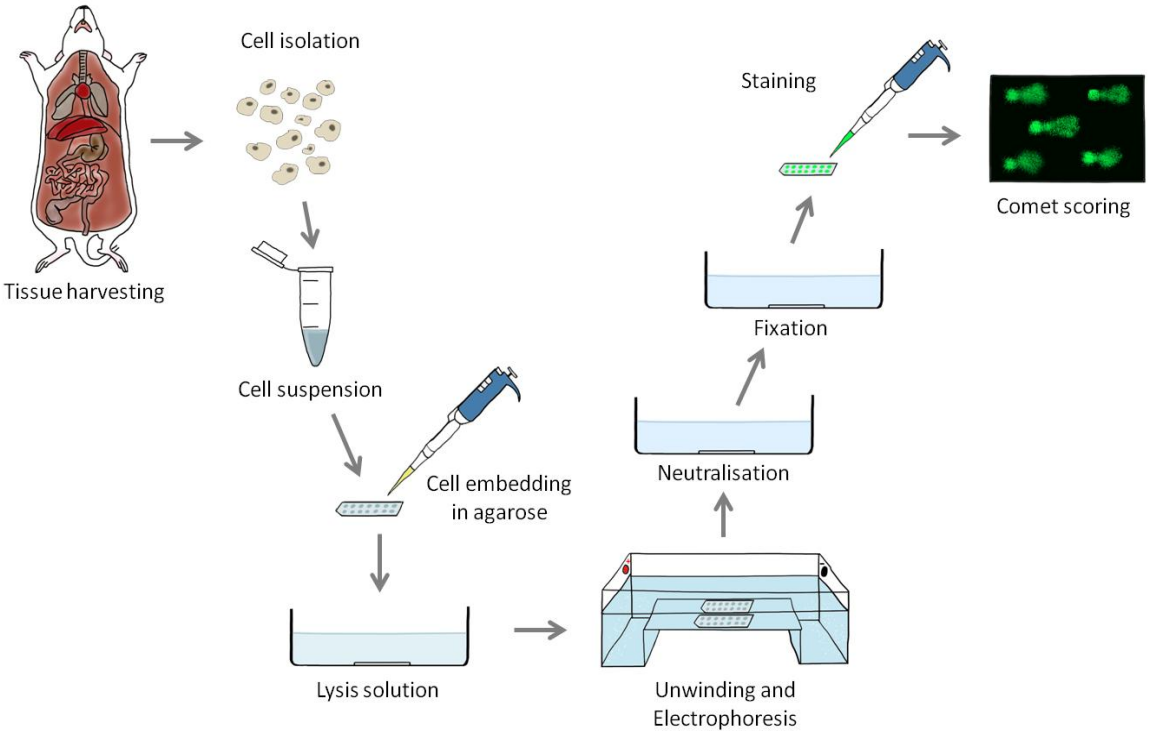


Figure 11. Steps involved in the performance of the alkaline comet assay *in vivo*.

- Effect of sample preparation time

One factor that is known to have an impact on the level of DNA damage is the time elapsed since the harvesting of the tissue from the animal until slide preparation (embedding single cells in agarose). It is reported on the OECD guideline (OECD TG 489, 2014) that this process should be done as quick as possible, ideally within 1 hour, to ensure a minimal inter-sample variation and to minimize the background DNA damage to allow the observation of appropriate positive and negative control responses.

Considering that in the first studies the sample time process exceeded 1 hour we decided to eliminate the step involving the cell counting. The average cell concentration obtained in the experiments we had performed up to this moment (N=3) was used to establish a fixed volume of cell dilution and buffer (final volume of 1 mL). Moreover, an additional cell dilution was prepared from the previous one (1:5) to guarantee a concentration that allowed the correct visualization of cells under the microscope (in case of 1:1 dilution was too concentrated).

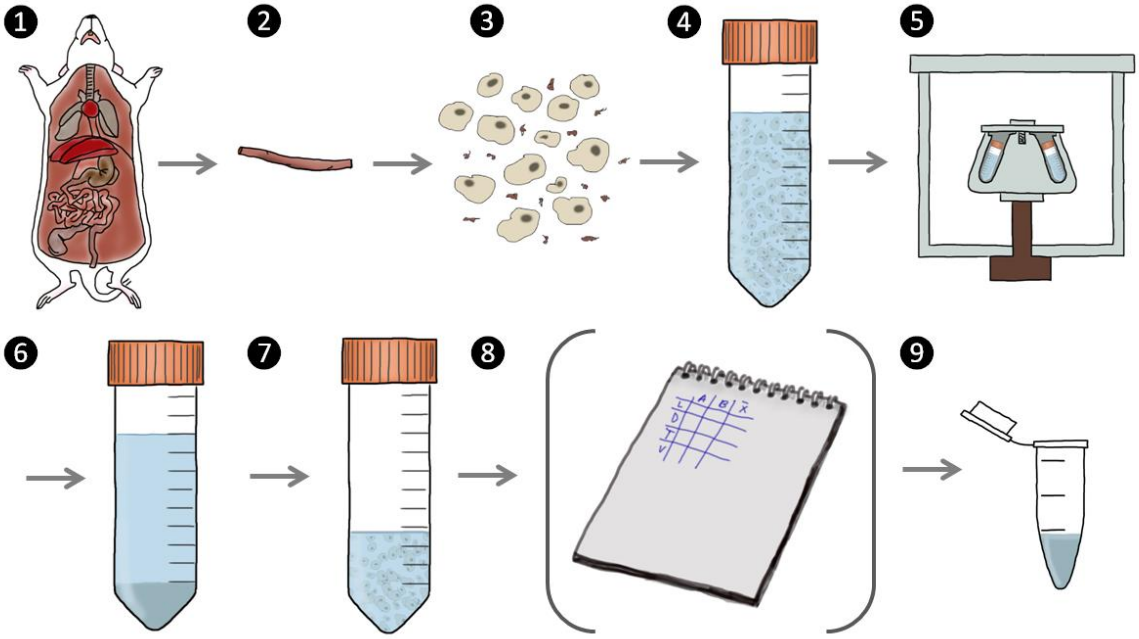


Figure 12. Cell dilution preparation steps. ① Animal sacrifice ② Tissue harvesting ③ Cell isolation ④ Cell suspension in PBS ⑤ Centrifugation (x2) ⑥ Removal of supernatant ⑦ Resuspension of the pellet in 2mL of PBS ⑧ Cell counting ⑨ Preparation of cell dilution with final concentration of $2{,}5 \cdot 10^5$ cells/mL.

3.2.3. Evaluation of the genotoxicity of SiNPs with comet assay

Once the finally adopted comet assay protocol was validated for *in vivo* samples, the genotoxicity of silica nanoparticles after a short-term oral administration in mice was evaluated by using the following methodology.

3.2.3.1. Animals

The animals used for the evaluation of the genotoxicity of SiNPs were *Swiss Webster* (strain RjOrl:SWISS) 4 weeks old female mice with an arrival body weight around 20-24 g. Animals complete a 7 day acclimatization period before starting the treatments. The total amount of animals was 120: 6 animals per condition (10 conditions) and 2 evaluation time points (60x2).

Mice housing conditions included controlled temperature (20 ± 2ºC) and relative humidity (55 ± 30%), 12:12 light:dark cycle. Mice were housed in groups of 4 in type II cages and with *ad libitum* access to food (protein rodent maintenance diet) and autoclaved water.

3.2.3.2. Test conditions

The 10 different conditions used for the assay are depicted in the following table:

TESTING GROUPS		DOSE	IDENTIFICATION CODE
VEHICLE CONTROL (CMC 0,2% (w/v) + Tween80® 1% (v/v))			CTRL
POSTIVE CONTROL (100mg/kg MMS)			MMS
SiNP Ø = 100 nm	Porous	Low dose (100 mg/kg)	100P LD
		High dose (1000 mg/kg)	100P HD
	Non-porous	Low dose (100 mg/kg)	100NP LD
		High dose (1000 mg/kg)	100NP HD
SiNP Ø = 300 nm	Porous	Low dose (100 mg/kg)	300P LD
		High dose (1000 mg/kg)	300P HD
	Non-porous	Low dose (100 mg/kg)	300NP LD
		High dose (1000 mg/kg)	300NP HD

- Vehicle control group

The control group of animals was treated with vehicle (solution used to disperse the nanoparticles and known to not produce relevant toxic effects. The results from the vehicle control group administered with CMC + Tween80® allowed evaluating the assay variability, the endogenous level of DNA damage, and the additional damage that may occur during sample preparation. Vehicle control animals and treated animals were handled in an identical manner.

- Positive Control

A group of animals treated with a substance producing DNA damage after oral administration was included in the test. The positive control substance used for inducing DNA strand breaks in the tissue of interest for the genotoxicity test was MMS (100 mg/kg in NaCl 0,9% (w/v) in miliQ water). The choice of using MMS was taken by a mutual accordance with a caLIBRAte partner Finnish Institute of Occupational Health (FIOH)[4], in order to use a positive control compound in both the comet assay and the micronucleus assay (OECD TG 489, 2014) (OECD TG 487, 2014).

- Silica nanoparticles

Silica nanoparticles (SiNPs) were synthesized at the National Research Centre for the Working Environment (Denmark) using Stöber method. The particles were methylated to minimize the release of ions (Si^{4+}) when the particle is in solution and consequently, minimize their dissolution. The particles were provided in powder form and for the administration, they were dispersed in carboxymethylcellulose (CMC sodium salt 0,2% (w/v) + Tween80 1% (v/v) in MiliQ water). CMC + Tween80® was chosen as a vehicle because it facilitates the homogenization of the dispersion due to the stabilizing effects of the CMC emulsion and the Tween80® surfactant properties.. Moreover, CMC-Tween80®

[4]The partners at Finnish Institute for Occupational Health (FIOH) perform the micronucleus assay in erythrocytes from peripheral blood, which is another genotoxicity assay that is performed using MMS as positive control.

is considered as a non-toxic vehicle and it is widely used for oral *in vivo* assays (i.e in Drug Discovery)(Thackaberry, 2013).

To disperse the NPs, an external force to overcome the Van Der Waals attractions was applied. Ultrasonication facilitates the dispersion of aggregates and agglomerates. The sonication was developed using a 750W sonifier (VCX750 Ultrasonic Cell Disrupter, SONICS Vibracell, USA) and a tappered microtip (diameter 3 mm; SIGMA-ALDRICH REF:Z192740-1EA). Sonication was performed for 5 minutes at 20% amplitude. The process was repeated if by visual inspection the sonication was not able to resuspend all the material from the bottom of the vial or if material is floating on the surface of the suspension.

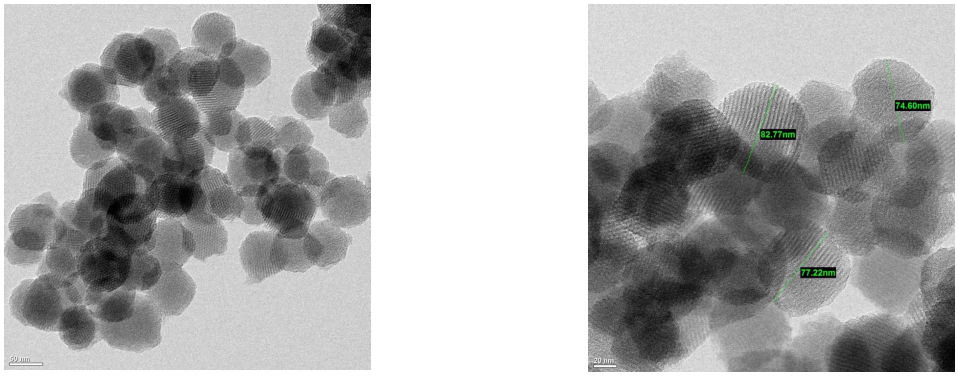


Figure 13. TEM images of porous silica 100 nm diameter nanoparticles (100P). Images ceded by the National Research Centre for the Working Environment (Denmark).

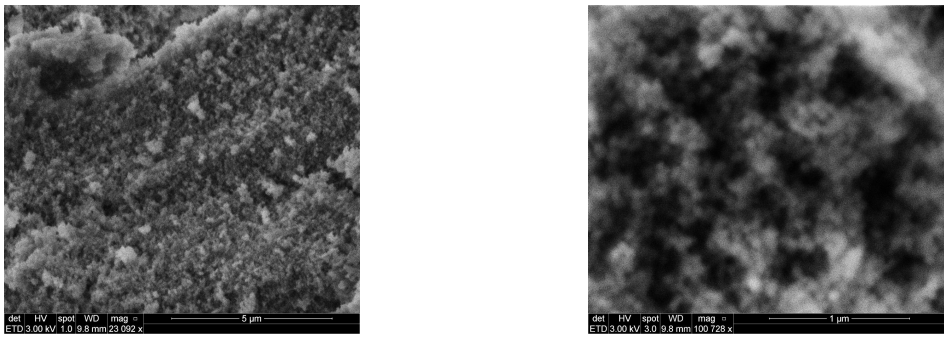


Figure 14. SEM images of porous silica 100 nm diameter nanoparticles (100P). Images ceded by the National Research Centre for the Working Environment (Denmark).

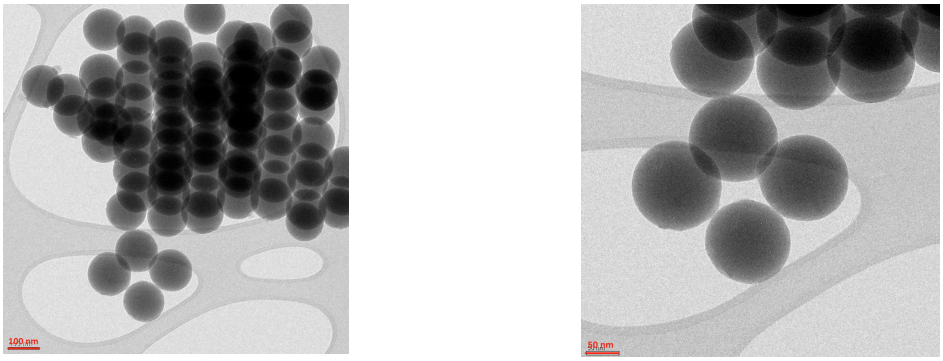


Figure 15. TEM images of non-porous silica 100 nm diameter nanoparticles (100NP). Images ceded by the National Research Centre for the Working Environment (Denmark).

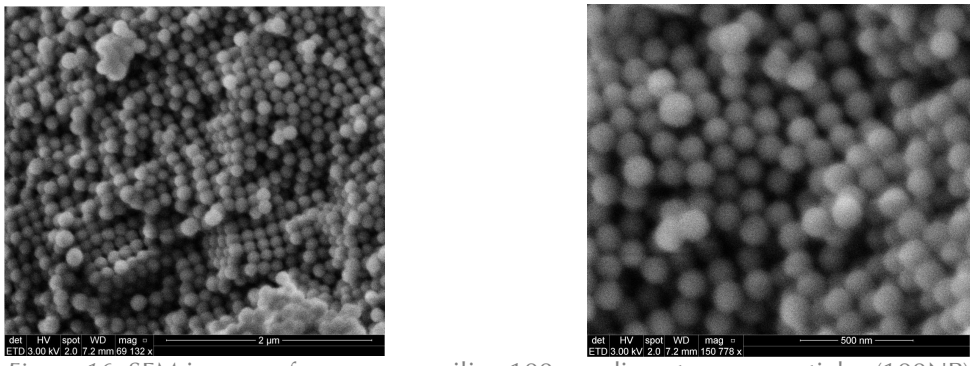


Figure 16. SEM images of non-porous silica 100 nm diameter nanoparticles (100NP).
Images ceded by the National Research Centre for the Working Environment (Denmark).

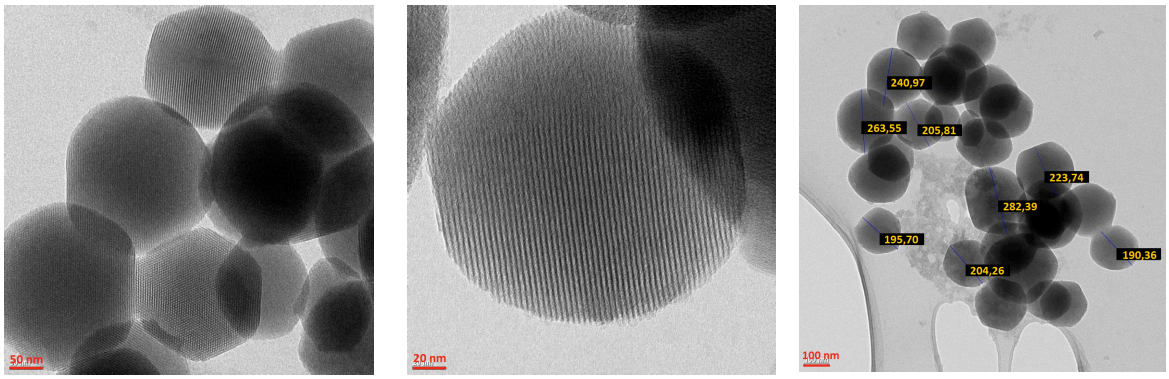


Figure 17. TEM images of porous silica 300 nm diameter nanoparticles (300P).
Images ceded by the National Research Centre for the Working Environment (Denmark).

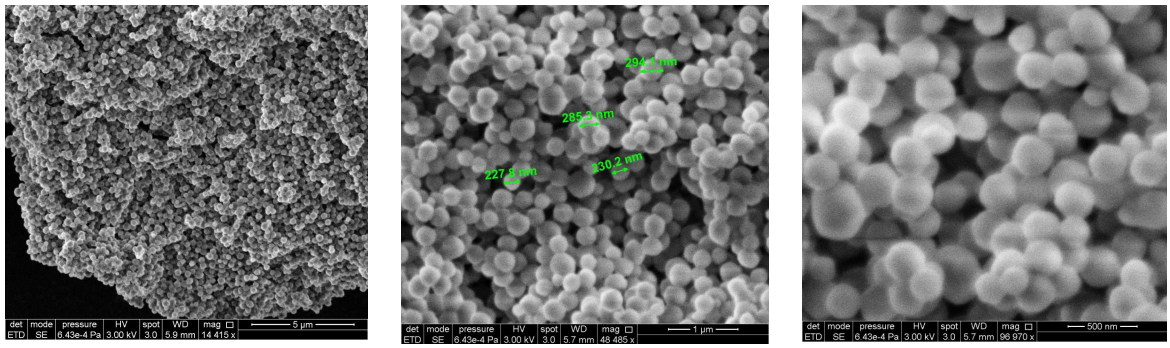


Figure 18. SEM images of porous silica 300 nm diameter nanoparticles (300P).
Images ceded by the National Research Centre for the Working Environment (Denmark).

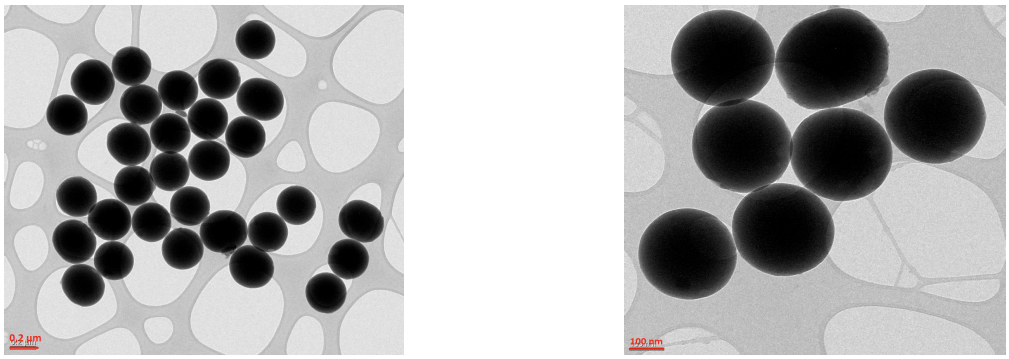


Figure 19. TEM images of non-porous silica 300 nm diameter nanoparticles (300NP).
Images ceded by the National Research Centre for the Working Environment (Denmark).

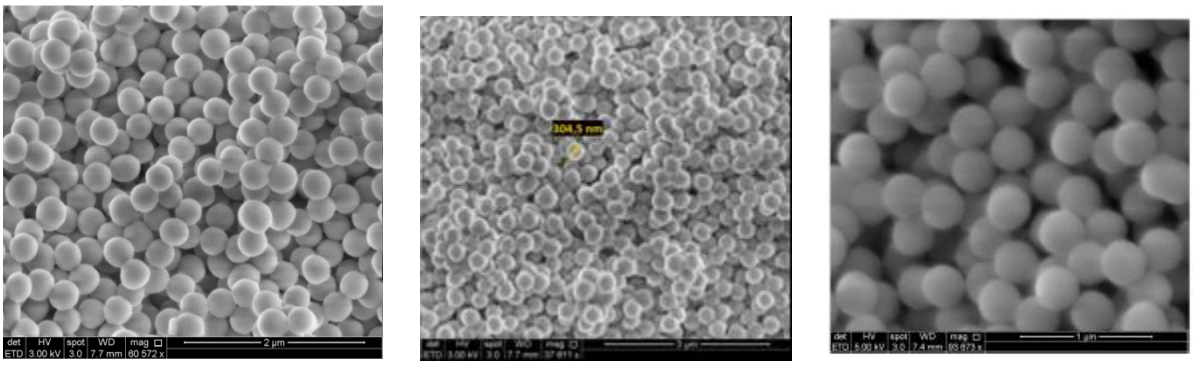


Figure 20. SEM images of non-porous silica 300 nm diameter nanoparticles (300NP). Images ceded by the National Research Centre for the Working Environment (Denmark).

3.2.3.3. Treatment schedule

The treatment schedule was designed following a test method that is gaining acceptance in the scientific community for the *in vivo* testing of inhalation toxicity of nanomaterials: the short-term inhalation study (STIS). In the STIS protocol, animals are administered for five consecutive days with 14- or 21-day post-exposure observation (Landsiedel *et al.*, 2014). Therefore, a similar design, but in our case for an oral exposure, was used.

Mice were orally administered during 5 consecutive days (with intervals of 24 h) and samples were collected 24 hours after the last administration for the non-recovery group (day 6) and 21 days after administration for the recovery group (day 26). Eight sacrifices per day for the whole set of experiments were performed (sets of 2 animals with intervals of 1 hour). However, only tissues from six animals were used for the purpose of this project due to time constraints (see Figure 21). Three doses of MMS were administered every 24 hours during two days and 2 - 3 hours before the sacrifice.

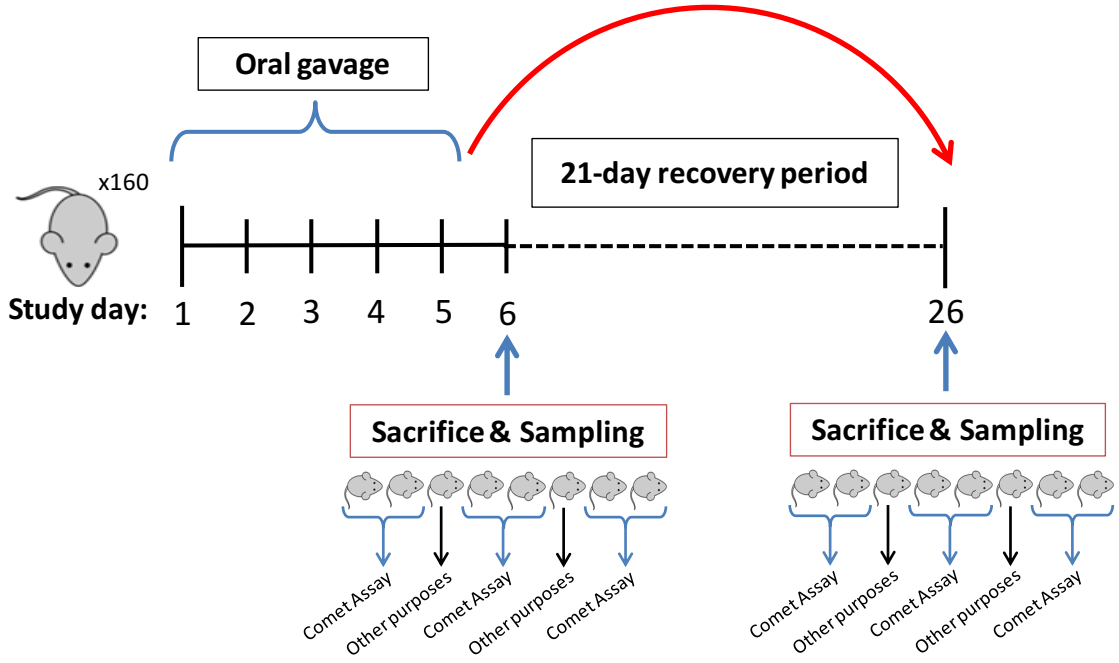


Figure 21. Treatment schedule of the assay.

3.2.3.4. Dose

- Dose levels

Two different doses for each SiNP were orally administered: a low dose (LD) of 100 mg/kg and a high dose (HD) of 1000 mg/kg. The decision of using these doses is based on previous studies about genotoxicity of orally administered SiNPs (Dekkers *et al.*, 2013). The 1000 mg/kg dose was selected as a tentative dose able to induce toxic effects and the 100 mg/kg was selected as a tentative dose not producing toxic effects. Animals corresponding to the positive control group (MMS) were orally administered at 100 mg/kg.

- Administration of doses

The volume recommended by oral gavage administration is 10 mL per kilogram of body weight. Considering the selected doses and the administration volume, NPs were prepared at 10 mg/mL and 100 mg/mL corresponding to the low and the high dose respectively.

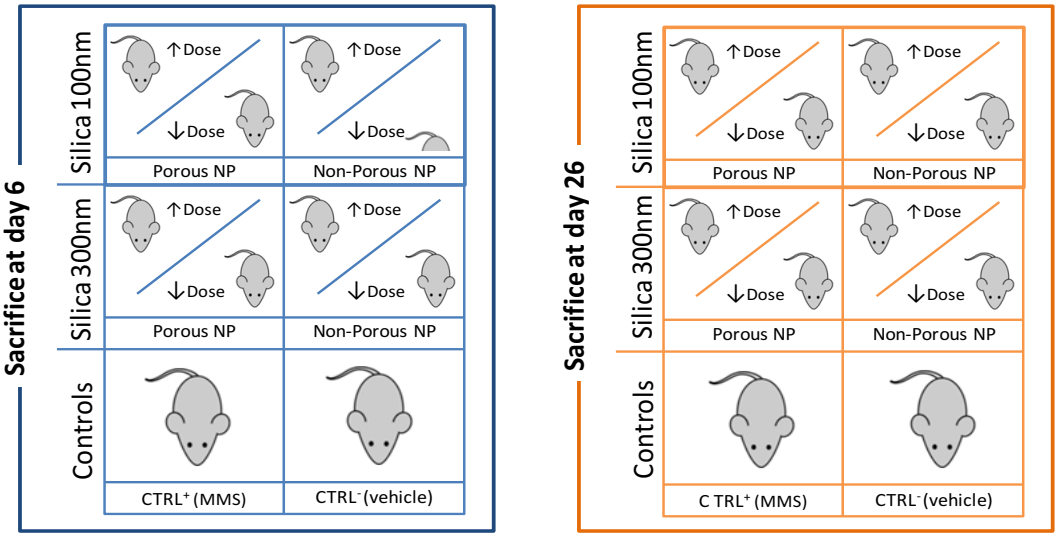


Figure 22. Graphical representation of the different conditions. Each experimental group has 6 animals.

3.2.3.5. Sacrifice

Mice were initially anesthetized with isofluorane and after loss of consciousness and hind paw withdrawal response, 0,5 to 1 mL of whole blood were drawn by cardiac puncture (blood extraction for other project purposes). After that, they were sacrificed by CO_2 asphyxiation to proceed with the opening of the abdominal cavity to harvest and dissect the gastrointestinal tract.

3.2.3.6. Preparation of specimens

The preparation of the cell suspension from tissue is described in point 1.2 from alkaline comet assay section 3.2.1.

3.2.3.7. Preparation of GelBond® film slides

Once the cell suspension was ready (adjusted to a target theoretical concentration of $2{,}5x10^5$ cells/mL), two dilutions were prepared to be embedded in agarose: dilutions 1:1 (C1) and 1:5 (C2). Six animals a day were sacrificed in three sets of 2 every hour (see Figure 21) and three slides containing 6 rows and 2 columns of drops were filled every day. For a correct orientation of the GelBond® film (due to its transparency appearance), the vertex were cut in different combinations (Figure 23).

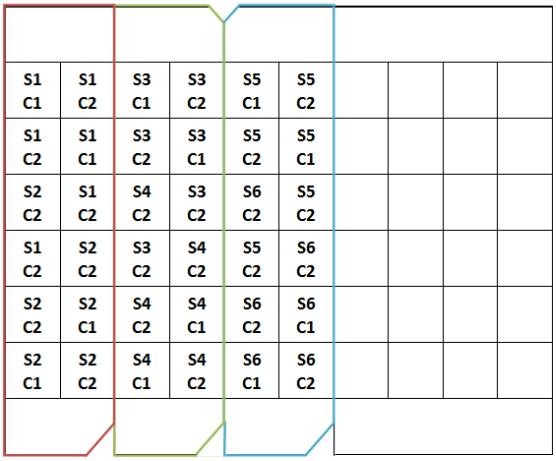


Figure 23. GelBond® template for setting of agarose drops. "S" refers to sample and "C" refers to concentration (C1 is 1:1 and C2 is 1:5). The cuts at the vertex provide information about which samples are contained in the slide (red: slide 1, green: slide 2 and blue: slide 3).

The rest of steps (lysis, unwinding, electrophoresis, neutralization and fixation) of the assay are explained in the alkaline comet assay methodology section 3.2.1 following the modified comet assay setup.

3.2.3.5. Statistical analysis of Comet tail intensity data

All statistical analyses were performed using Graphpad Prism® software (Prism 6.02 for Windows, La Jolla, USA).

Day 6

Before carrying out the significance test, a normality test (e.g. Shapiro-Wilk test) to check the continuity of the data and a posterior test for homogeneity of variance (e.g. Barlett's test) was realized. To perform the normality test, following the literature, the positive control group (MMS) was omitted from the statistical model due to the obvious variability with the other groups (McNamee and Bellier, 2015). Once it was determined that the data was homogeneous, pairwise comparisons back to vehicle were performed using Dunnett's test adjusting 5% significance threshold for individual comparisons (see Figure 24)(OECD 451 2010).

Day 26

In this case, the data did not show a normal distribution and, therefore, the data was logarithmically transformed. A new normality test with the logarithmic data and a posterior test for homogeneity of variance (e.g. Barlett's test) were realized. Once it was determined that the log transformed data was normally distributed and homogeneous, pairwise comparisons back to vehicle were performed using Dunnett's test adjusting 5% significance threshold for individual comparisons (see Figure 24) (OECD TG 451, 2010).

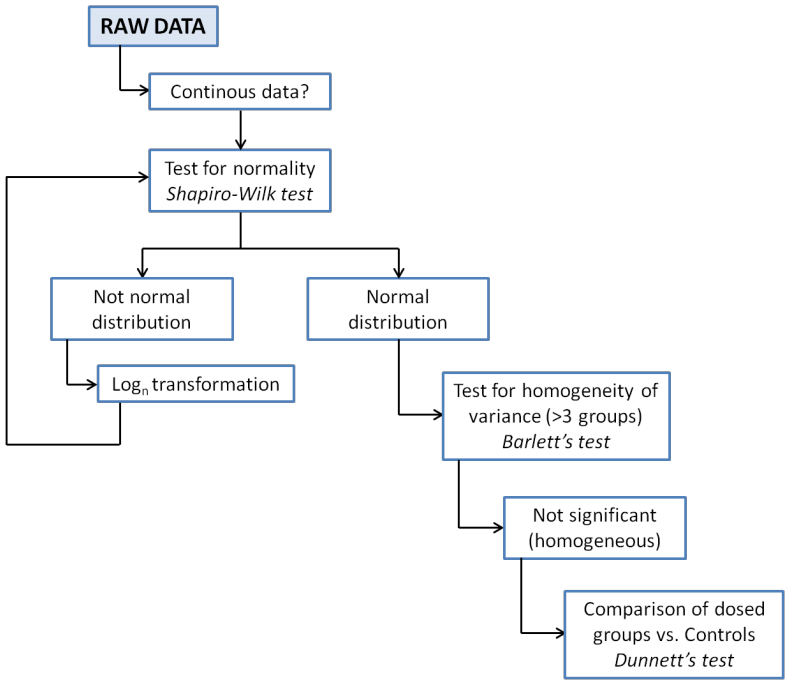


Figure 24. Significance analysis flowchart for tail intensity raw data.

3.2.4. Study of the aggregation state of SiNP dispersions used for oral administration with TEM

The tested SiNPs (100NP, 100P, 300NP and 300P) were provided in powder form and for the administration they were mixed with the vehicle (CMC sodium salt 0,2% (w/v) + Tween80 1% (v/v) in MiliQ water) and sonicated to disperse the aggregates and agglomerates and facilitate their homogenization (5 min 20% amplitude with a microtip-style homogenizer probe of 3 mm diameter[5]). To check the dispersion achieved after sonication, TEM imaging was performed. The microscope used was a JEOL JEM-2100 HT operated at an accelerating voltage of 200 kV.

From the initial sonicated particle dispersion at 100 mg/mL (corresponding to the 1000 mg/kg dose), serial vortexed dilutions using vehicle were performed until obtaining a final concentration of 50 µg/mL. For the preparation of TEM grids (Formvar/carbon-coated 200 mesh Cu grids; Ted Pella Inc.) a drop of 20 µL of the final concentration was placed on the grid, the NPs were led to settle for 2 minutes and let air dry.

[5] Sonication is repeated if by visual inspection the sonication was not able to resuspend all the material from the bottom of the vial or if material is floating on the surface of the suspension.

4. RESULTS AND DISCUSSION

4.1. Validation of the in vivo comet assay setup

As mentioned before, the *in vivo* comet assay that was conducted in this project was part of a bigger experiment that included additional toxicological endpoints. To fit the comet assay within the logistics of the whole experiment, some adaptations where included to basically allow the performance of the protocol within two working days.

4.1.1. In vitro validation of selected assay conditions

Before starting to manipulate fresh tissue samples from mice, the sensitivity of the adapted comet assay protocol was evaluated *in vitro* by assessing the genotoxic effect of MMS and EMS (positive controls). The images in Figure 25 show some examples of the results obtained for this set of experiments *in vitro*.

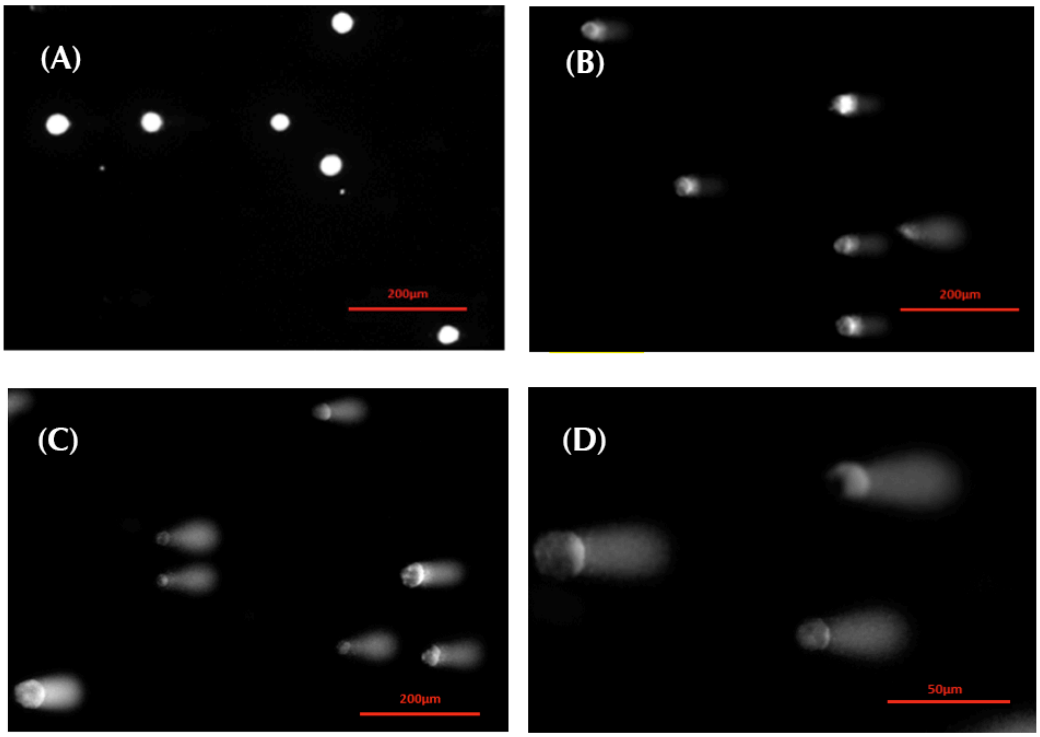


Figure 25. Cell images corresponding to (A) CTRL cells taken at 10x, (B) cells exposed to EMS (40 µg/mL) taken at 10x, (C) cells exposed to MMS (200 µg/mL) taken at 10x and (D) cells exposed to MMS (200 µg/mL) taken at 40x.

Just by observing the images, it is clearly visible that cells exposed to the positive control substances (i.e. EMS and MMS, Figure 25B, 25C and 25D) show tails (comets) whereas the control cells (non-exposed cells, Figure 25A) are seen as dots without tails. This observation reflects the principle of the comet assay: after the electrophoresis, undamaged DNA run as an entity while damaged DNA is broken in smaller parts that run faster displaying the comet shape. Moreover, from the microscopy images, the intensity of the comet tails present in MMS exposed cells (Figure 25C) are higher than the ones in the

EMS exposed ones (Figure 25B). However, it should be noted that the MMS concentration was higher than the EMS concentration.

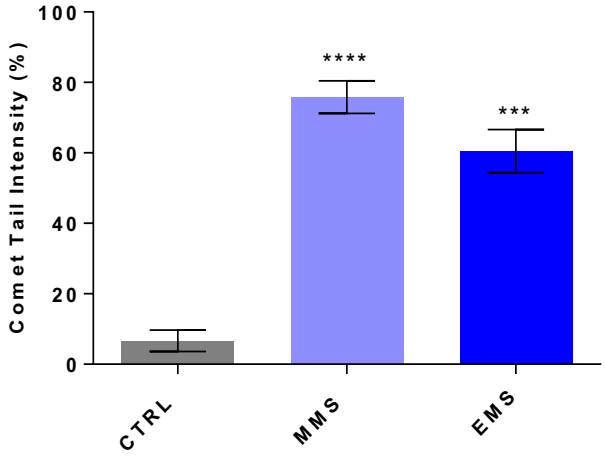


Table 2. DNA tail intensity (%) in control cells (CTRL) and in cells exposed to MMS and EMS. Values are medians of all evaluated cells per condition (>93).

	EXP 1	EXP 2	EXP 3	Mean	SEM
CTRL	5,32	2,20	12,4	6,65	3,03
MMS (40 µg/mL)	83,3	76,7	67,5	75,8	4,61
EMS (200 µg/mL)	71,9	50,9	58,6	60,5	6,11

After quantification of the % of tail intensity data (see Visualization and Scoring of comets in section 3.2.1), a one-way ANOVA was used to check whether the differences between the tested substances and the control were statistically significant. Consistently with the qualitative assessment of the microscopy images described above, the test showed that there was a significant difference between the non-exposed cells and the cells exposed either to MMS and EMS ($p<0,0001$ and $p=0,0004$ respectively). These results support the sensitivity of the adapted comet assay protocol *in vitro.*

4.1.1. *In vivo* validation of selected assay conditions

The following step was performed to demonstrate that the protocol would be sensitive also *in vivo,* which markedly differs from the *in vitro* protocol on the sample preparation step. MMS was chosen as positive control substance (after agreement with another partner from the project in charge of performing another genotoxic assay). The images in Figure 26 show some examples of the results obtained for this set of experiments *in vivo*.

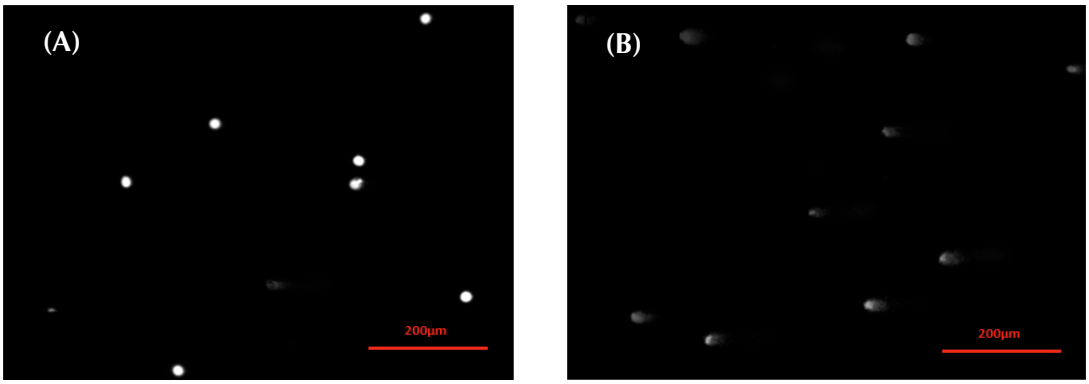


Figure 26. Cell images corresponding to (A) CTRL animal cells taken at 10x and (B) cells from animals exposed to MMS (100µg/mL) taken at 10x.

In the same manner as in the *in vitro* assay, from the images of the *in vivo* assay, it is visible that the animals administered with the positive control test substance (MMS, Figure 26B) present a much higher amount of cells showing tails compared to the vehicle control group animals (Figure 26A).

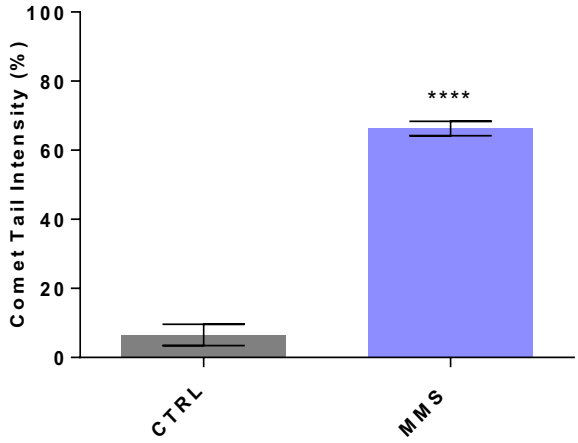


Table 3. DNA tail intensity (%) in control animals (CTRL) and in animals exposed to MMS. Values are medians of all evaluated cells per condition (>80).

	EXP 1	EXP 2	EXP 3	Mean	SEM
CTRL	4,22	12,64	2,73	6,53	3,09
MMS (100mg/kg)	68,67	62,14	68,02	66,28	2,08

The two-tailed nonparametric Student t-test showed that there is a significant difference in the % tail intensity between the non administered mice and MMS orally administered mice ($p<0,0001$). The results also showed a relatively small variability between assays and the magnitude of the DNA damage was similar to that observed *in vitro*.

The % of tail intensity values of both the vehicle control and the positive control groups obtained in the *in vivo* assay are slightly higher than the ones obtained in other experiments from the literature. For instance, Tarantini *et al.* (2015) used MMS (80 – 100 mg/kg) as positive control in an oral genotoxicity study and reported values of % of tail intensity ranging from 2 – 3.5 % for CTRL and 30 – 45 % for MMS in rat duodenum. Interlaboratory and interassay differences in % tail intensities are common and indeed, according to the OECD guideline TG 489, the ranges of % of tail intensity values for the negative control must be established for each tissue and specie at every laboratory.

4.1.2. Effect of sample preparation time

To evaluate if the time employed between the animal sacrifice and the embedding of cells in agarose affected the background level of DNA damage in our testing conditions, we compared % of tail intensity from control samples with different processing times. Two statistical tests were realized: a test to evaluate if there was any correlation between time and comet tail intensity, and a test to compare tail intensities among different processing times.

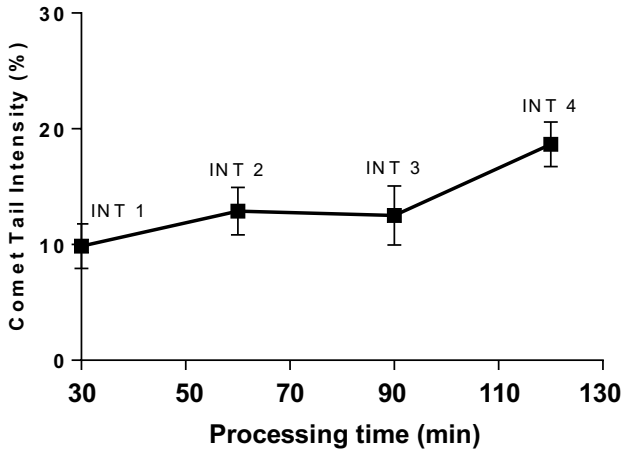


Table 4. % of tail intensity represented as mean of medians from three independent experiments. Time (in minutes) is counted from the sacrifice of the animal until the embedding of isolated cells in agarose. (R^2= 0,8203 and P (two-tailed)= 0,0943).

	Mean	SEM
INTESTINE 1 (t=30min)	9,86	1,92
INTESTINE 2 (t=60min)	12,88	2,06
INTESTINE 3 (t=90min)	12,50	2,57
INTESTINE 4 (t=120min)	18,67	1,94

From the graphic a trend towards increased % of tail intensity with increasing processing times is observed. Nevertheless, the correlation test (Pearson's test) showed no significant correlation between time and % comet tail intensity (P=0,0943). Afterwards, an ANOVA test was performed to detect differences in DNA damage between samples.

Table 5. One-way ANOVA test results performed with Tukey's post-hoc.

Tukey's multiple comparisons test	95% CI of diff,	Significant?	Adjusted P value
INTESTINE 1 vs. INTESTINE 2	-12,70 to 6,666	no	0,7548
INTESTINE 1 vs. INTESTINE 3	-12,33 to 7,044	no	0,8186
INTESTINE 1 vs. INTESTINE 4	-18,49 to 0,8771	no	**0,0753**
INTESTINE 2 vs. INTESTINE 3	-9,306 to 10,06	no	0,9992
INTESTINE 2 vs. INTESTINE 4	-15,47 to 3,896	no	0,2947
INTESTINE 3 vs. INTESTINE 4	-15,85 to 3,518	no	0,2509

The results from the one-way ANOVA with Tukey's post-hoc test demonstrate that there are not significant differences between any of the samples. However, comparison of samples from intestine 1 and 4 show the lowest P value (P=0,0753) and the highest difference in % tail intensity is achieved. This result together with the previous data in the literature (Hobbs *et al.*, 2015) (OECD TG 489, 2014) pointing to the need of reducing the preparation time lead us to decide to reduce the time lapse to 1 hour (or less). To this aim, the cell counting was not performed and instead the average cell concentration in the three experiments performed up to that moment was used.

Table 6. Calculated values for cell dilution based on data from previous cell countings.

	EXP 1	EXP 2	EXP 3	Mean	SEM
Cell concentration (cells/mL)	1,2E+06	5,6E+05	8,1E+05	8,8E+05	2,1E+05
Volume of cells (mL)				0,36	
Volume of PBS (mL)				0,64	

To account for possible variations in the real cell concentration values obtained during the final *in vivo* experiment, it was decided to prepare two working dilutions: the dilution that would lead to the theoretically target concentration of $2{,}5x10^5$ cells/mL with 0,4 mL cells + 0,6 mL of PBS (values rounded off the means), and the dilution 1:5 consisting of 0,2 mL of the previous dilution and 0,8 mL of PBS.

4.2. Evaluation of the genotoxicity of SiNPs with comet assay

Once the validation of the comet assay protocol was achieved both using *in vitro* and *in vivo* samples, it was applied to address the main objective of this project: the evaluation of the genotoxicity of SiNPs *in vivo* after a short-term oral administration. However, prior to this step, the aggregation state of SiNPs was studied by TEM imaging reproducing the same conditions used for the oral administration.

4.2.1. Study of the aggregation state of SiNP dispersions used for oral administration with TEM

In order to check how the nanoparticles were dispersed in the vehicle, TEM images were performed as defined in section 3.2.4 from Methods.

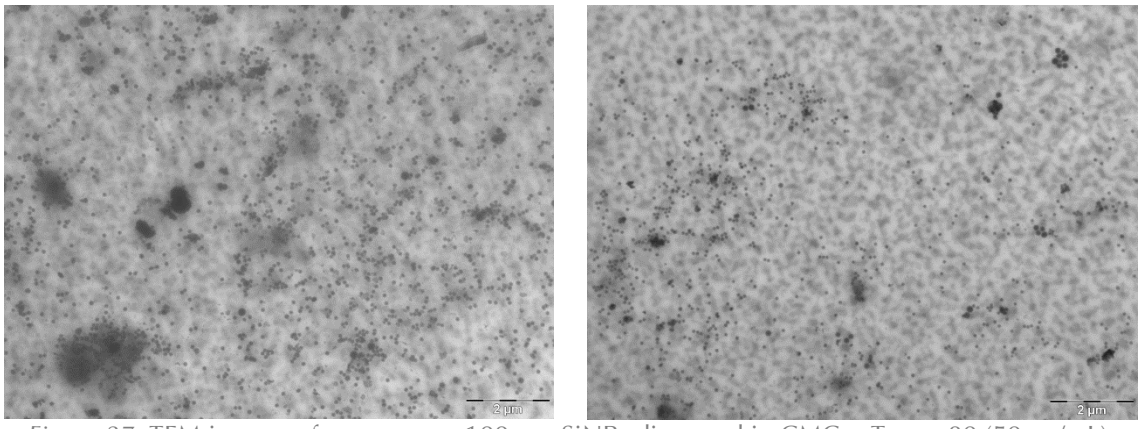


Figure 27. TEM images of non-porous 100 nm SiNPs dispersed in CMC + Tween80 (50 µg/mL).

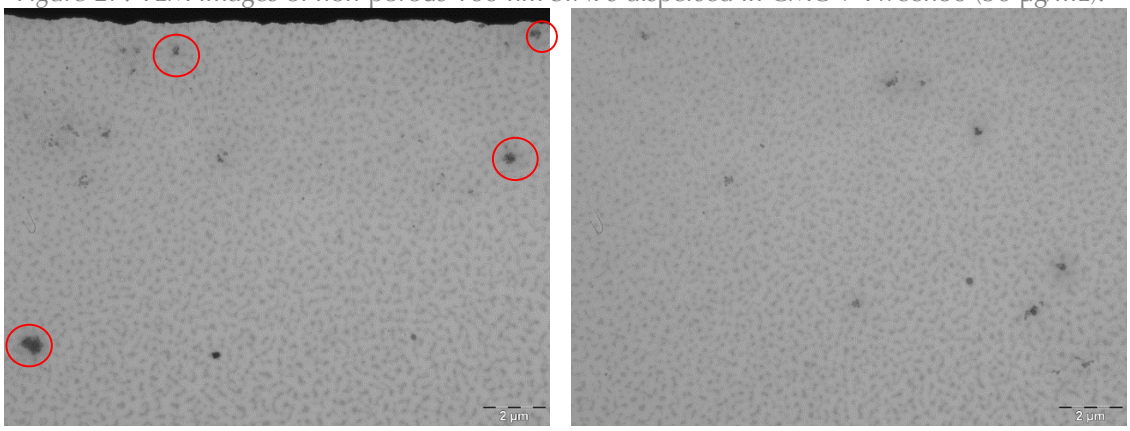


Figure 28. TEM images of porous 100 nm SiNPs dispersed in CMC + Tween80 (50 µg/mL).

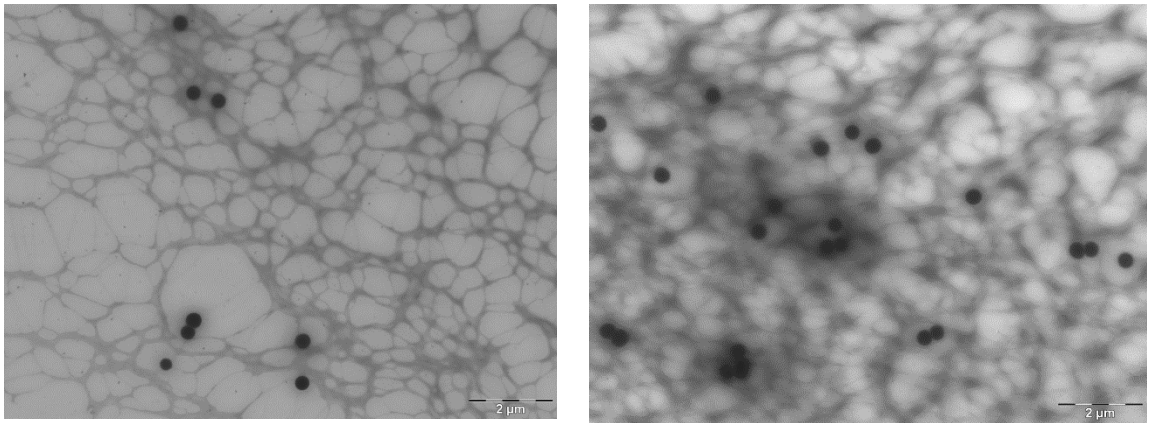


Figure 29. TEM images of non-porous 300 nm SiNPs dispersed in CMC + Tween80 (50 µg/mL).

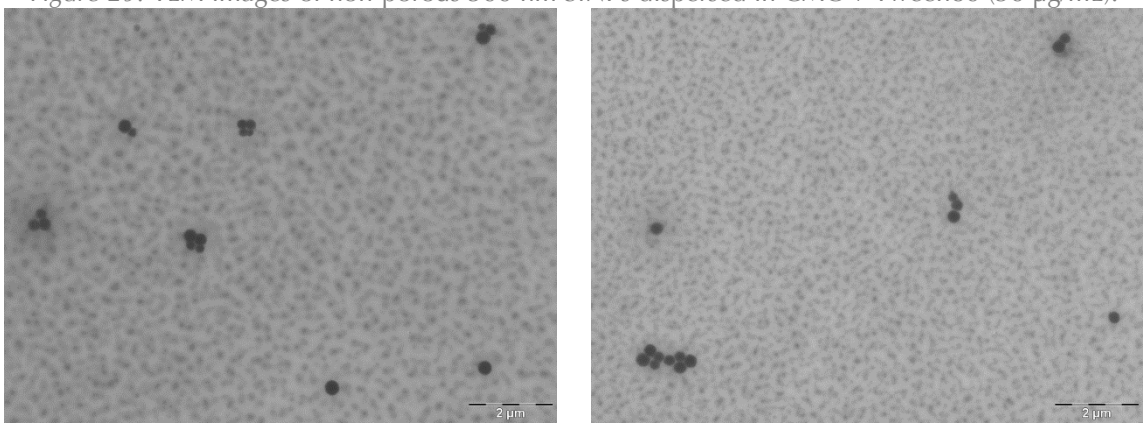


Figure 30. TEM images of porous 300 nm SiNPs dispersed in CMC + Tween80 (50 µg/mL).

From the TEM images it is visible that the nanoparticles tended to form aggregates/ agglomerates (>1 µm) but, at the same time these agglomerates were homogeneously distributed throughout the CMC net. Moreover, in Figure 29 from non-porous 300 nm SiNPs mostly all nanoparticles were found individually. Figure 30 show that the agglomerates of porous 300 nm SiNPs consist in small groups of 3 – 4 nanoparticles.

The aggregation state of NPs depends on their reactivity (related to their SSA). Suttiponparnit *et al.* (2011) proved that at fixed pH, increasing the particle surface area (SSA) led to a higher degree of agglomeration with TiO_2 nanoparticles (Suttiponparnit *et al.*, 2011). From our SiNPs SSA data (see Table "Nanomaterials" at Methods section), it was expected that the porous nanoparticles would agglomerate more than the non-porous ones.

TEM images have been helpful to get an idea about the distribution of the nanoparticles in the CMC. For TEM evaluation, aliquots of the different NPs suspensions were diluted and dried. During the drying process on the TEM grid, NPs may aggregate, and the drying of CMC could also lead to changes in their distribution. For that reason, the images may not reflect the real state of the particles during administration. Dynamic Light Scattering (DLS) would have been a better approach to directly evaluate aggregate size distribution in the suspension (although major dilutions would have also been necessary) but, due to the presence of detergent micelles (Tween80), it was not viable (data not shown).

4.2.2. Evaluation of effects at day 6

The first evaluation of the genotoxic effects of the SiNPs was at day 6. The animals corresponding to this group were administered by gavage during five consecutive days and sacrificed after 24 hours from the last administration. The preparation of the cell suspension and their embedding in agarose lasted less than one hour to minimize the effects of inter-sample variations and reduce the DNA damage. The data corresponding to the results from the assay at day 6 is depicted in Table 7.

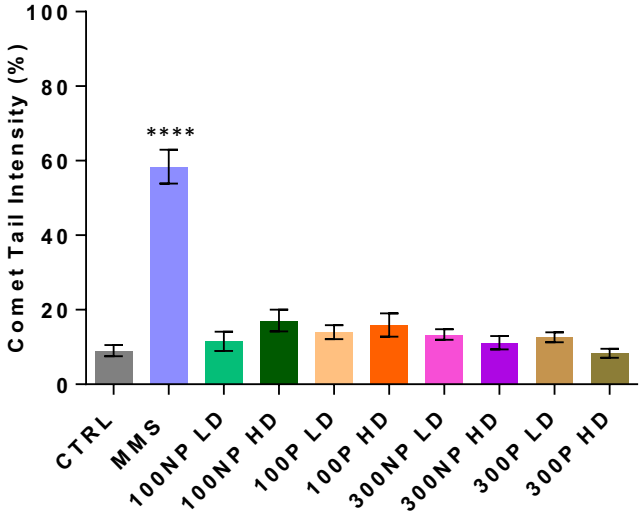


Table 7. Statistic values for all conditions at day 6, mice without recovery period. N=6 for all conditions except for 300nm porous nanoparticle at high dose (300P HD) in which one sample did not show analyzable results (N=5).

	CTRL	MMS	100NP LD	100NP HD	100P LD	100P HD	300NP LD	300NP HD	300P LD	300P HD
Mean of medians	9	58,4	11,5	17,1	14	15,9	13,4	11,2	12,6	8,3
SEM	1,5	4,6	2,6	2,9	1,9	3,1	1,4	1,8	1,3	1,1

The graphic and Table above show the means of medians of all the scored cells (6 animals per condition with ~100 cells scored per animal) and their associated SEM. From the numerical and graphical results obtained from the evaluation at day 6, it is visible that all conditions present very similar values of % tail intensity except for the positive control group (MMS), which shows clearly elevated values.

Table 8. Multiple comparisons test for all conditions at day 6, mice without recovery period.

Dunnett's multiple comparisons test	Mean Diff.	95% CI of Diff.	Significant?	Summary	Adjusted P Value
CTRL vs. MMS	**-49,37**	**-59,07 to -39,66**	**Yes**	****	**< 0,0001**
CTRL vs. 100NP LD	-2,498	-12,20 to 7,21	No	ns	0,99
CTRL vs. 100NP HD	-8,091	-17,80 to 1,61	No	ns	0,14
CTRL vs. 100P LD	-4,97	-14,67 to 4,73	No	ns	0,63
CTRL vs. 100P HD	-6,893	-16,60 to 2,81	No	ns	0,28
CTRL vs. 300NP LD	-4,366	-14,07 to 5,34	No	ns	0,76
CTRL vs. 300NP HD	-2,144	-11,85 to 7,56	No	ns	0,99
CTRL vs. 300P LD	-3,586	-13,29 to 6,12	No	ns	0,89
CTRL vs. 300P HD	0,7242	-9,454 to 10,90	No	ns	0,99

After completing the multiple comparisons with Dunnett's test, the results showed that only MMS presented significant difference respect to vehicle control group.

4.2.3. Evaluation of effects at day 26

The second evaluation of the genotoxic effects of the SiNPs was at day 26. The animals corresponding to this group were also administered by oral gavage during five consecutive days but then sacrificed after 21 days from the last administration. The data corresponding to the results from the assay at day 26 is depicted in Table 9.

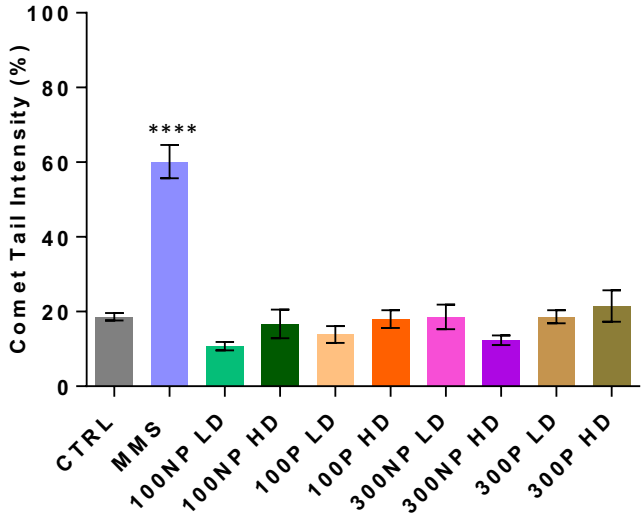


Table 9. Statistic values for all conditions at day 26, mice experienced a 21-day recovery period. N=6 for all conditions except for the positive control (MMS) in which one sample did not show analyzable results (N=5).

	CTRL	MMS	100NP LD	100NP HD	100P LD	100P HD	300NP LD	300NP HD	300P LD	300P HD
Mean of medians	18,6	60,2	11,7	16,7	13,8	18	18,6	12,3	18,6	20,1
SEM	2,5	10	2,7	9,4	5,5	5,8	3,2	8,1	10,4	4,3

The graphic and Table above show the numerical values corresponding to the means of medians for % tail intensity of all the scored cells (6 animals per condition with ~100 cells scored per animal) and the associated SEM. From these results, it is visible that the positive control is markedly higher than the rest of conditions. In this case, the % tail intensity of the vehicle control group is twice the obtained at day 6 whereas the positive control (MMS) remains almost equal.

Equally to the statistics performed with the data from day 6, it was performed for day 26 to evaluate the differences between conditions.

Table 10. Multiple comparisons test for all conditions at day 26, mice with 21-day recovery period.

Dunnett's multiple comparisons test	Mean Diff,	95% CI of diff,	Significant?	Summary	Adjusted P Value
CTRL vs. MMS	**-41,53**	**-52,94 to -30,12**	**Yes**	****	**< 0,0001**
CTRL vs. 100NP LD	7,88	-3,00 to 18,75	No	ns	0,26
CTRL vs. 100NP HD	1,92	-8,96 to 12,79	No	ns	0,99
CTRL vs. 100P LD	4,78	-6,10 to 15,66	No	ns	0,78
CTRL vs. 100P HD	0,61	-10,26 to 11,49	No	ns	0,99
CTRL vs. 300NP LD	0,06	-10,81 to 10,94	No	ns	> 0,99
CTRL vs. 300NP HD	6,31	-4,57 to 17,19	No	ns	0,49
CTRL vs. 300P LD	0,03	-10,85 to 10,91	No	ns	> 0,99
CTRL vs. 300P HD	-2,88	-13,76 to 7,995	No	ns	0,98

The multiple comparisons with Dunnett's test showed that the only group that presented a statistically significant difference respect to the vehicle control was the positive control (MMS).

Tarantini *et al*. (2015) investigated the genotoxic effects caused by four synthetic SiNPs at three different doses administered during three consecutive days by oral gavage in rats. They performed the alkaline comet assay in different tissues to test the genotoxicity among other tests. They concluded that no differences between the % tail intensities from the tissues of animals administered with SiNPs and the ones from the negative control were observed. Their positive control (100 mg/kg MMS) induced a statistically significant increase in DNA damage in all organs investigated. The mean of medians % of tail intensity for the biggest NP (24.76 ± 17.7 nm) at the highest dose (20 mg/kg) was around 12% (Tarantini *et al.*, 2015). Moreover, Maser *et al*. (2015) tested the genotoxicity with the alkaline comet assay caused by SiNPs of two different sizes in rats by single intratracheal administration (10 mg/m³). They concluded that no relevant genotoxic effects were observed in the lung cells for none of the SiNPs and that only the positive control (EMS) generated significant differences compared to the vehicle control. The biggest SiNP (55 nm) presented a mean relative % tail intensity of 12.8%. Therefore, our results are consistent with the ones from previous experiments.

The choice of performing the genotoxic evaluation at day 26 even though no genotoxicity was found at day 6 was based on previous studies showing that adverse effects of nanoparticles may aggravate after the end of the exposure period. For instance, Landsiedel *et al*. (2014) concluded that SiNPs multifocal macrophage aggregates were observed in the respiratory tract immediately after the exposure period that aggravated towards a slight multifocal inflammation during the 21-day post exposure period (recovery period), suggesting a long lasting effect of SiNPs.

4.2.4. Evaluation of the role of porosity and SSA

The presence of pores is directly related to an increase in specific surface area (SSA), and therefore, to the NP reactivity. Therefore, the presence of pores in NPs could have

implications related to genotoxic effects. In this study no DNA damage was observed for any of the tested particles (% tail intensity values within the background DNA damage levels). However, as an exercise, we showed the type of analyses that would have been used to evaluate the effect of porosity if genotoxicity had been observed.

To do that, different tests can be performed. We decided to carry out a two-tailed unpaired Student t-test (95% confidence interval) comparing the same conditions (NP diameter, dose and sacrifice day) for porous and non-porous pairs as shown in Table 11.

Table 11. Two-tailed unpaired Student t-test (95% confidence interval) results for comparison of the effect of porosity.

		P value	P value summary	Significant?
100NP LD vs. 100P LD	**DAY 6**	0,45	ns	no
	DAY 26	0,25	ns	no
100NP HD vs. 100P HD	**DAY 6**	0,79	ns	no
	DAY 26	0,78	ns	no
300NP LD vs. 300P LD	**DAY 6**	0,69	ns	no
	DAY 26	0,99	ns	no
300NP HD vs. 300P HD	**DAY 6**	0,23	ns	no
	DAY 26	0,06	ns	no

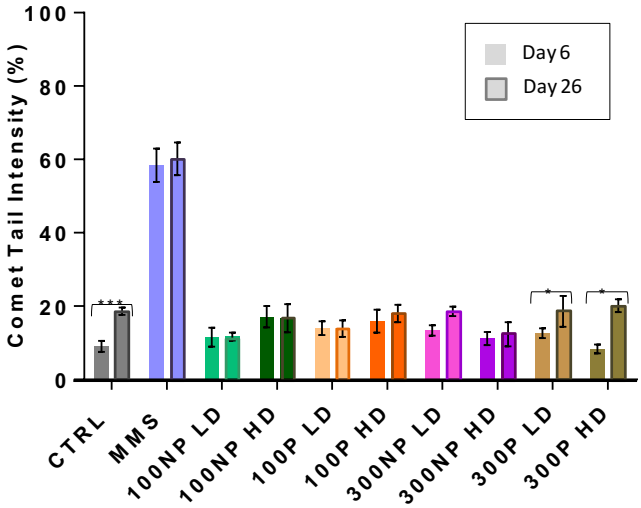
The results of the Student t-test showed that there were no significant differences between porous and non-porous nanoparticles (either at day 6 or day 26).

Moreover, another statistical that can be performed would be a correlation between % tail intensity and total SSA administered (the later parameter can be calculated multiplying SSA (m^2/g) by the quantity of NP administered in grams).

4.2.5. Evaluation of time after last administration

As nanoparticles are very reactive and have a tendency to aggregate in some tissues, a potential harmful effect can be triggered at the DNA level. However, cellular machinery has the ability of repairing DNA of damaged cells (Friedberg, 2003).

Despite no differences were found for any of the NPs when compared to the vehicle control group in any of the time points evaluated, as an exercise, we showed the type of analyses that would have been used to evaluate the effect of recovery if genotoxicity had been observed.



Graph 1. Comet tail intensities (in %) for all conditions at day 6 and day 26. The corresponding values are depicted in Tables 7 and 9.

A statistical significance analysis of % tail intensity between day 6 and day 26 was performed to check the effect of recovery time. Each condition at the two time points was compared with a two-tailed Student t-test (95% confidence interval).

Table 12. Two-tailed unpaired Student t-test (95% c.i) results to check the effect of recovery.

	P value	P value summary	Significant?
CTRL	**0,0004**	***	**Yes**
MMS	0,79	ns	No
100NP LD	0,78	ns	No
100NP HD	0,93	ns	No
100P LD	0,96	ns	No
100P HD	0,61	ns	No
300NP LD	0,18	ns	No
300NP HD	0,62	ns	No
300P LD	**0,02**	*	**Yes**
300P HD	**0,02**	*	**Yes**

The results showed that no statistical significant differences were remarkable for any of the conditions except for the vehicle control, and 300 nm porous nanoparticles groups, both at low and high doses. A significant increase in % tail intensity values was observed in the vehicle control group, therefore, the differences observed in the animals exposed to the SiNPs between the two time points could not be clearly evaluated.

One possible explanation for the increase of % tail intensity at day 26 could be the age. Hamilton *et al.* observed a significant increase in 8-oxo-2-deoxyguanosine (oxo8dG) levels (indicator of oxidative damage) in DNA with age in all tissues and strains of rodents studied (Hamilton *et al.*, 2001). However, these studies were performed with ages up to 24 months, whereas other studies spamed a much shorter period. Another possibility for explaining this increase is the inherent intervariability of DNA damage levels among samples.

5. CONCLUSION

The *in vitro* alkaline comet assay implementing the selected assay conditions (affecting the lysis and fixation steps) and the two positive controls showed results that lead to conclude that the adapted setup was sensible for detecting DNA damage.

The assay was applied *in vivo* to assess whether it was able to adequately detect differences between a vehicle control and a positive control chemical compound (MMS) administered in rodents by the oral route. The pre-established ranges of % tail intensity in jejunum samples from vehicle control and positive control were established around 2 – 13 % and 60 – 70 % respectively. The *in vivo* values were within these ranges: 6.5% (vehicle control, CTRL) and 66% (positive control, MMS). Therefore, after performing the significance analysis between the groups, we concluded that the assay with the selected conditions was also able to adequately discriminate between vehicle and positive controls *in vivo*.

The effect of sample preparation time was checked by comparing the % tail intensities from cells of jejunum samples with different processing times. Although there were no significant increases in % tail intensity among different processing times, to stick to the OECD guideline (TG 489) (OECD TG 489, 2014) recommendations to avoid an increase in % tail intensity related to sample preparation time, it was concluded that the cell counting *in situ* would be eliminated from the process.

It should be noted that most of the effects observed in the *in vivo* studies were obtained using unrealistically high doses of NPs. However, none of the tested conditions presented significant differences in % tail intensity compared to the vehicle control group, except for the positive control. These results lead to conclude that after 5 days of oral gavage, any of the tested nanoparticles generated detectable genotoxic effects in jejunum by the comet assay.

Equally to the results obtained for the samples from the assay at day 6, results from the tested conditions at day 26 also showed no statistically significant differences compared to the vehicle control group, except for the positive control (MMS). In this scenario, it is concluded that the tested nanoparticles did not produce genotoxic effects detectable by the comet assay at day 26 either.

The role of porosity on genotoxicity could not be evaluated in the study due to the lack of genotoxicity detected for any of the tested conditions.

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