Short-Term Inhalation Study in Rats for Testing of Nanomaterials

Date

28th September 2012

Version

1.1 English

Content

Short-Term Inhalation Study in Rats for Testing of Nanomaterials 1
1 Summary 3
2 Introduction 3
3 Initial Considerations 4
4 Description of the Method 4
4.1 Selection of Animal Species 4
4.2 Preparation of Animals 5
4.3 Animal Husbandry 5
4.4 Inhalation Chambers 5
5 Toxicity Study 5
6 Exposure Conditions 7
6.1 Administration of Concentrations 7
6.2 Particle-Size Distribution 7
6.3 Test Article Preparation 7
7 Monitoring of Exposure Conditions 7
7.1 Chamber Airflow 7
7.2 Chamber Temperature and Relative Humidity 8
7.3 Test Article: Nominal Concentration 8
7.4 Test Article: Actual Concentration 8
7.5 Test Article: Particle Size distribution 9

8 Clinical Observations 9
9 Deposition and Translocation 10
10 Clinical Pathology 10
11 Gross Pathology and Organ Weights 11
12 Histopathology 12
13 Data and Reporting 13
13.1 Data 13
13.2 Test report 13
14 References 14

1 Summary

This SOP has been designed to characterize toxicity of nanomaterials applied by the inhalation route following short-term exposure in order to provide data on effects, their recovery/progression and tissue burdens within 28 day. Major emphasis is put on the effects on the respiratory tract. Groups of male rats are head-nose exposed to aerosols generated by dry or liquid dispersion techniques for 6 hours a day on five consecutive days to the test article at three or more concentration levels and filtered air (negative control) or the, where applicable, vehicle (vehicle control), respectively. After 3 and 23 days of post exposure observation examination of bronchoalveolar lavage fluid (BALF) and histopathological examination of respiratory tract are performed. If the material is analytically ascertainable, the amounts of the test material in the lung and in the mediastinal lymph nodes (and other tissues) can be determined.

2 Introduction

1. The increased use of engineered nanomaterials in the past several years has compelled the scientific community to investigate the potential hazards of these unique and useful particles. When materials reach the nano-scale, they often display an increased reactivity relative to the bulk compound. New approaches for testing and new ways of thinking about current materials are necessary to provide safe workplaces and products. The available test methods for testing of chemicals need to be adapted to meet the information requirement for nanoparticle toxicity.
2. Nanoparticles, soluble in water and/or biological fluid, are not purpose of this guideline. Although the bioavailability and the toxicokinetics of soluble nano-particles of a chemical may be somewhat different to those with large particle sizes, they are supposed to exert the intrinsic biological activity of the chemical after dissolution and transport to the target tissues.
3. This guideline describes a method for the characterization of adverse effects and tissue burdens following short-term inhalation exposure (five days) to a test article for a total of 28 days. Using sensitive parameters, it provides early evidence on respiratory tract effects which might also occur by long-term inhalation exposure to aerosols of nanomaterials. The data derived from this short-term study may be used for prioritization, grouping and preliminary risk assessments. The data can also provide information on the selection of concentrations for longer term studies such as the 90-day inhalation toxicity study. Def-

initions used in the context of this guideline can be found in Annex I and OECD GD 39 (2).

3 Initial Considerations

An ILSI Research Foundation/Risk Science Institute Nano-material Toxicity Screening Working Group has previously developed the elements of a screening strategy for the hazard identification of engineered nanomaterials (3). A fundamental conclusion of this report is that adequate characterization of nanomaterials is required.

The following physical-chemical properties are required:

1. Size distribution (e.g. dynamic laser scattering measurement)
2. Agglomeration state
3. Shape
4. Crystal structure
5. Chemical composition
6. Surface properties (area, chemistry, charge)
7. Porosity
8. Solubility

One lot of the test article should be used, if possible, throughout the duration of the study, and a research sample should be stored under conditions that maintain its purity and stability. Prior to the start of the study, there should be a characterization of above mentioned parameters. If it is not possible to use one lot, lot-to-lot variability based on characterisation data should be provided.

4 Description of the Method

4.1 Selection of Animal Species

Healthy young adult rodents of commonly used laboratory strains should be employed. The preferred species is the rat. Due to their higher ventilation rate, male rats are considered to be more susceptive than females concerning local effects on the respiratory tract. Therefore, the more sensitive male rats should be used to present a worst case scenario.

4.2 Preparation of Animals

On the day of first exposure, animals should be young adults 7 to 9 weeks of age to assure full lung maturation. Body weights should be within 20 % of the mean weight of each test group. The animals are marked for individual identification and acclimatized to laboratory conditions.

4.3 Animal Husbandry

Animals should be randomly selected to the test groups and individually identified to facilitate observations. The temperature of the animal maintenance room should be 22 ± 3 °C. The relative humidity should ideally be maintained in the range of 30 to 70 %. Before and after exposures, animals may be caged in groups per concentration, but the number of animals per cage should not interfere with clear observation of each animal and should minimize losses due to cannibalism and fighting. The animals should be acclimated to the restraining tubes by exposing to conditioned air in the exposure tube at least two days before they are exposed to the test item. Conventional laboratory diets may be used, except during exposure, accompanied with an unlimited supply of municipal drinking water. Dust-free bedding material should be used to prevent effect caused by dust fraction of the bedding. Lightening should be artificial, the sequence being 12 hours light and 12 hours dark.

4.4 Inhalation Chambers

Dynamic nose-only inhalation systems (the term “nose-only” includes head-only, nose-only or snout-only) should be used. Details of different nose-only inhalation systems are addressed in GD 39 (2).

5 Toxicity Study

The study consists of a minimum of three concentration levels, and a concurrent negative and/or vehicle control as needed. Each test group contains eight rats per examination time point. Animals are exposed for 5 consecutive days. Main group animals of each concentration are sacrificed on the third day after the last exposure day (study day 8). Recovery groups should be used to observe reversibility, persistence, or delayed occurrence of toxicity after an exposure-free period of approximately 3 weeks following the last exposure (e.g. on study day 28). If technically feasible the content of the test item in the lung and in the medi-

astinal lymph nodes should be determined analytically in additional satellite groups animals as indicated in table 1.

Study day	1	2	3	4	5	6	7	8	9-27	28
Study phase	x	x	x	x	x	R	R	R	R	R
Examinations and their time points					E+L			L		E+L

x: head/nose exposure for 6 hours a day

R: recovery period

E: gross necropsy, organ weights and histopathology (3 animals/ time point and concentration group)

optionally lung burden (3 animals/time point and concentration group)

L: bronchoalveolar lavage (5 animals/ time point and concentration group)

Figure Study design

The target concentrations selected should identify the target organ(s) and demonstrate a clear concentration-response:

- The high concentration level should result in toxic effects but not cause lingering signs of lethality which would prevent a meaningful evaluation.
- The intermediate concentration level should be spaced to produce a gradation of toxic effects between that of the low and high concentration.
- The low concentration level should produce little or no evidence of toxicity.

Concurrent control animals should be handled in identical manner to the test group animals except that they are exposed to filtered air rather than test article. When vehicle is used to assist in generating the test atmosphere, a vehicle control group, instead of negative control group, should be included in the study. Water should be used as the vehicle whenever possible. Surface-active agents might be used, if there is no other way to produce stable particle suspensions for spraying, but their use is strongly discouraged because their intrinsic respiratory tract toxicity might complicate the interpretation of study results. For this reason, surface-active agents used should be considered in the vehicle control at a concentration that is

comparable as its concentration in the test groups. If surface active agents is not used, a vehicle control group with water may be not necessary.

6 Exposure Conditions

6.1 Administration of Concentrations

Animals are exposed to the test article for 6 hours per day on five consecutive days. Feed and water should be withheld during the exposure period.

Animals are exposed to aerosols of the test article generated by dry (dust) or liquid dispersion techniques. The selected concentrations, and the physical form should resemble conditions prevailing during the handling and use of the test article.

6.2 Particle-Size Distribution

Particle sizing should be performed for all aerosols and for vapours that may condense to form aerosols. Nanoparticles are manufactured to have very small particle sizes, thus the aerodynamic particle sizes of the aerosols might be lower than the current recommendation for exposure atmospheres on mass median aerodynamic diameter (MMAD) of 1 to 3 µm (4) and the geometric standard deviation might be higher than the currently recommended range of 1.5 to 3.0.

6.3 Test Article Preparation

Ideally, the test article should be tested unchanged. If it is necessary to use a vehicle to generate an appropriate test article concentration and particle size, water should be given preference. Whenever a test article is suspended in a vehicle, constancy and homogeneity of its atmospheric concentration should be ensured.

7 Monitoring of Exposure Conditions

7.1 Chamber Airflow

The flow of air through the exposure chamber/system should be carefully controlled, continuously monitored, and recorded at least hourly during each exposure. The monitoring of the test atmosphere concentration (or its stability over time) is an integral measurement of all dynamic parameters and provides an indirect means to control all relevant dynamic inhalation parameters. Therefore, the frequency of measurement of air flows may be reduced to

one single measurement per exposure per day, if real time concentration monitoring is applied. Oxygen concentration should be at least 19 % and carbon dioxide concentration should not exceed 1 %. If there is reason to believe that this standard cannot be met, oxygen and carbon dioxide concentrations should be measured. If measurement on the first day of exposure show that these gases are at proper levels, no further measurements should be necessary.

7.2 Chamber Temperature and Relative Humidity

Chamber temperature should be maintained at 22 ± 3 °C. Relative humidity in the animals´ breathing zone should be monitored continuously and recorded hourly during each exposure where possible. The relative humidity should ideally be maintained in the range of 30 to 70 %, but this may either be unattainable or not measurable due to test article interference with the determination method.

7.3 Test Article: Nominal Concentration

If feasible, the nominal exposure concentration should be calculated and recorded. The nominal concentration is the mass of test article consumed during test atmosphere generation divided by the total volume of air passed through the exposure system. For nano-particle testing, the nominal concentration is not used to characterize the animal s' exposure. Therefore the nominal concentrations need not to be calculated, especially not, if particle separation or air dilution systems are used.

7.4 Test Article: Actual Concentration

The actual concentration is the test article concentration as sampled from the animals´ breathing zones in an inhalation system. For the non-volatile single-component nanoparticles, the actual concentrations can be obtained by non-specific gravimetric filter analysis. For multi-component aerosol concentration may also be determined by gravimetric analysis. However, this requires analytical data which demonstrate that the composition of airborn is similar to those starting material.

The exposure atmosphere should be held as constant as possible. A monitoring device, such as an aerosol photometer should be used to demonstrate the stability of the exposure concentration over time. Actual concentrations should be measured at least 3 times during each

exposure day for each exposure level. Individual concentration measurements should deviate from the mean chamber concentration by no more than ± 20 %.

If a vehicle other than water is used, the atmosphere concentration of the vehicle should be determined in a proper way (e.g. gaschromatography).

7.5 Test Article: Particle Size distribution

The particle size distribution of aerosols should be determined once during the study for each concentration level by using a cascade impactor. The total mass concentration obtained by particle size analysis should be within reasonable limits (20 %) of the mass concentration obtained during concentration control analysis.

To enhance the resolution of measurements in the range of visible particle sizes, in addition to the cascade impactor an optical particle sizer (OPC) may be used.

To further characterize the presence of free nano-particles in the inhalation atmospheres a scanning mobility particle sizer (SMPS) should be used.

8 Clinical Observations

The animals should be observed before, during and after the exposure on each exposure day, or more frequently when indicated by the response of the animals to treatment. The observation of animals is often hindered by the use of head nose inhalation systems, thus emphasis of observation should lie after daily exposure and before the subsequent exposure.

All observations are systemically recorded with individual records being maintained for each animal. It is important to note that poor appearance immediately following head-nose exposure may be not a substance-related clinical finding but induced by the exposure method.

Cage-side observations should include changes in the skin and fur, eyes and mucous membranes; changes in the respiratory pattern and any other signs of toxicity.

Individual animal weights should be recorded shortly before the first exposure (day 1) and on the day of last exposure (day 5) prior to exposure, once a week during the recovery period

and at study termination. Food consumption should by measured in parallel to the body weight. Measurement of water consumption may be omitted.

9 Deposition and Translocation

To examine the deposition of the test substances in the lung and its translocation ability, the test substance should be determined quantitatively in the lung, in the lung-draining lymph nodes as well as in the blood. The study director may choose to assess additional organs. The measurements should be performed in at least 3 animals per concentration group (including the control) sacrificed immediately after the last exposure and after the recovery period. For multi-component test substance, the major compound should be determined in parallel to one of the minor compound, to assess whether disproportion has taken place.

10 Clinical Pathology

Bronchoalveolar lavage should be performed 3 days after the last exposure day and after a recovery period of 3 weeks after end of exposure. Blood sampling should be performed shortly before lung lavage.

The lavage procedures published are highly variable. Depending on the procedure (e.g. instillation volume, instillation-retraction cycles, massage, excision of lung) the absolute value of the parameters differ strongly from each other. Therefore, it is worthwhile to have the lavage technique largely standardized in order to obtain reproducible and comparable results. Example: For the lavage 0.9 % sodium chloride is used. The targeted lavage volume per instillation is 20 to 22 mL/kg rat. After exsanguination, the lungs are lavaged in situ via a tracheal cannula. Two lavage cycles (instillation – retraction) are performed, the recovered volumes of bronchoalveolar lavage fluid (BALF) pooled for further investigations. Finally the lavaged whole lung (without trachea) may be weighed and homogenized and prepared for further examination. Because of the above mentioned diversity of the lavage technique, more attention should be paid on the relative change to the concurrent control rather than an inter-laboratory comparison of the absolute. Moreover, by providing positive control (e.g. exposed to quartz dust) data the validity of a procedure can be proved.

The bronchoalveolar lavage fluid (BALF) and the homogenate of the lavaged lung tissue should be examined for a set of parameters to characterize the pulmonary toxicity of the test article.

The following parameters should be examined in the BALF: recovered volume, total protein concentration, lactate dehydrogenase (LDH), alkaline phosphatise (ALP), N-acetyl-β-D-glucosaminidase (NAG) and γ-glutamyltransferase (GGT) activity as well as total cell counts and cell differential analysis of cytospin preparations.

In order to better characterize the lung toxicity of the compound other constituents may be measured in the BALF additionally, such as mediators reflecting inflammatory cell infiltration (e.g., Monocyte chemoattractant protein 1 (MCP-1), Interleukin 8 (IL-8)) or cell proliferation markers (e.g., Macrophage colony stimulating factor (M-CSF), Osteopontin). It is recommended to measure some further mediators in the lung tissue homogenate, such as immune system modulating mediators (e.g., Interleukin I α (IL-1 α), Tumour necrosis factor α (TNFα)).

In order to assess the presence of systemic inflammation the following parameters should be measured in the blood: total white blood cell (WBC) and differential leukocyte count, haptoglobin and C-reactive protein.

Other parameters of haematology and clinical chemistry as well as urine analysis are not required on a routine basis, but may be measured when deemed useful based on expected or observed toxicity.

11 Gross Pathology and Organ Weights

The animals designated for histopathological examinations should be killed by exsanguination from the abdominal aorta and vena cava under appropriate anesthesia and subjected to complete gross necropsy. As poorly soluble nano-particles are not expected to exert pronounced systemic toxicity, lethality during the study or removal of animal for humane reasons are highly unlikely. If there are any premature deaths, those animals should be subjected to complete gross necropsy. If a necropsy cannot be performed immediately after a dead animal is discovered, the animal should be refrigerated at a temperature low enough to minimize autolysis. Necropsy should be performed as soon as possible, normally within a day or two. All gross pathology changes should be recorded for each animal with particular attention to any changes in the respiratory tract.

Table 3 lists the organs and tissues that should be preserved for histopathological examination. The preservation of further organs and tissues and any other organs and tissues is at the discretion of the study pathologist. The bolded organs should be trimmed and weighed wet as soon as possible after dissection to avoid drying. Tissues and organs should be placed in 10 % buffered formalin or another suitable fixative as soon as necropsy is performed, and fixed at least for 48 hours prior to trimming.

Table

Organs and Tissues Preserved During Gross Necropsy (including number of sections)

Nasal cavity (4 levels, transverse)

Pharynx (including the nasopharyngeal duct, transverse)

Larynx (3 levels including the base of the epiglottis, transverse)

Trachea (2 levels including 1 longitudinal section through the carina and 1 transverse section)

Lung (all lobes at one level, including main bronchi)

Upper (cervical/submandibular) and lower (mediastinal/tracheobronchial/hilar) respiratory tract lymph nodes

Liver

Kidney

Spleen

Thymus

Brain

The lungs should be removed intact, weighed, and instilled with a suitable fixative (4).

12 Histopathology

A histopathological evaluation of the respiratory tract should be performed for animals of the control and high concentration groups, as well as for all animals which die or are sacrificed for animal well fare reasons. Particular attention should also be paid to organs with gross lesions and weight changes. The control and high concentration animals should be sacrificed after the last exposure. Organs with treatment-related findings should be examined in all concentration groups, as well as in the recovery group animals.

Sections should be collected from all lung lobes at one level, including main bronchi.

At least 4 levels of the nasopharyngeal tissues should be examined, one of which should include the nasopharyngeal duct (5, 6, 7, 8, 9) to allow adequate examination of the squamous, transitional (non-ciliated respiratory), respiratory (ciliated respiratory) and olfactory epithelium, and the nose associated lymphatic tissue (NALT, 10, 11). Three levels of the larynx should be examined, and one of these levels should include the base of the epiglottis (12). The trachea should be examined including one longitudinal section through the carina extrapulmonary bronchi and one transverse section bifurcation of the of the.

13 Data and Reporting

13.1 Data

Individual animal data on body weights, food consumption, clinical pathology (including lavage parameters), gross pathology, organ weights, and histopathology should be provided in tabular form. Summarized data on body weights, food consumption, and clinical pathology (including lavage) should show the number of animals used, main value and the standard deviation. For some parameters median and 5th/95th percentile may be given instead of main and standard deviation. In addition to the measured values, lavage parameters should also be presented as relative change to the concurrent control. All quantitative results should be evaluated by an appropriate statistical method. Any generally accepted statistical method may be used and the statistical methods should be selected during the design of the study.

Clinical observation data should be summarized in tabular form showing for each test group the number of animals used, the number of animals displaying specific signs of toxicity, a description and time course of toxic effects and reversibility. Necropsy and histological findings are to be summarized in a table presenting incidence and severity of any abnormalities.

13.2 Test report

The test report should include all information requirement of OECD 412.

Moreover, particle size distribution measured by SMPS should be reported for each concentration. If measurement of lung burden was performed, this information should be linked to the effects found.

14 References

1. ISO (International Standards Organization) (2007). Workplace atmospheres – Ultrafine, nanoparticle and nano-structured aerosols – Inhalation exposure characterization and assessment. Technical Report ISO/TR 27628. International Standard Organisation, Geneva, Switzerland.

2. OECD (2008) Draft Guidance Document on Acute Inhalation Toxicity Testing. Environmental Health and Safety Monograph Series on Testing and Assessment No. 39. Available: [http:\\www.oecd.org\dpci,emt\22\0,2340,en_2649_34377_1916054_1_1_1_1,00.html]

3. Oberdörster G., Maynard A., Donaldson K., Castranova V., Fitzpatrick J., Ausman K., Carter J., Karn B., Kreyling W., Lai D., Olin S., Monteiro-Rivere N., Warheit D., Yang H. (2005) A report from the ILSI Research Foundation/Risk Science Institute Nanomaterial Toxicity Screening Working Group. Principles for characterizing the potential human health effects from exposure to nanomaterials: Elements of a screening strategy. Part. I Fibre Toxicol. 2: 8

4. Whalan, J.E. and Redden, J.C. (1994). Interim Policy for Particle Size and Limit Concentration Issues in Inhalation Toxicity Studies. Office of Pesticide Programs, United States Environmental Protection Agency.

5. Dungworth D.L., Tyler W.S., and Plopper C.E. (1985). Morphological Methods for Gross and Microscopic Pathology (Chapter 9) in Toxicology of Inhaled Material, Witschi H.P. and Brain J.D. (eds.), Springer Verlag Heidelberg, pp. 229-258.

6. Young J.T. (1981) Histopathological examination of the rat nasal cavity. Fundam. Appl. Toxicol. 1, 309-312.

7. Harkema J.R. (1990) comparative pathology of the nasal mucosa in laboratory animals exposed to inhaled irritants. Environ. Health Perspect. 85, 231-238.

8. Wouterson R.A., Garderen-Hoetmer A. van, Slootweg P.J. and Feron V.J. (1994) Upper respiratory tract carcinogenesis in experimental animals and in humans. In: Waalkes

M.P. and Ward J.M. (eds.) Carcinogenesis. Target Organ Toxicology Series, Raven Press, New York, 215-263.

9. Mery S., Gross E.A., Joyner D.R., Godo M. and Morgan K.T. (1994) Nasal diagrams: A tool for recording the distribution of nasal lesions in rats and mice. Toxiclol. Pathol. 22, 353-372.

10. Kuper C.F., Koornstra P.J., Hameleers D.M.H., Biewenga J., Spit B.J., Duijvestijn A.M., Breda Vriesman van P.J.C. and Sminia T. (1992) The role of nasopharyngeal lymphoid tissue. Immunol. Today 13, 219-224.

11. Kuper C.F., Arts J.H.E. and Feron V.J. (2003) Toxicity to nasal-associated lymphoid tissue. Toxicol. Lett. 140-141, 281-285.

12. Lewis D.J. (1981) Mitotic indicies of Rat Laryngeal Epithelia. Journal of Anatomy 132(3), 419-428.

13. Ma-Hock L, Burkhardt S, Strauss V, Gamer A, Wiench K, van Ravenzwaay B, Landsiedel R (2008): Development of a short-term inhalation test in rats using nano-titanium dioxide as a model substance, Inhalation Toxicology, 21:102-118, 2009