

Proceedings of the 17th Conference about Laboratory Animals

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The 17th Conference about Laboratory Animals organized by the Czech Laboratory Animal Science Association (SVLZ) was held ashore Brno dam of the South Moravia, May 5 – 7, 2014 in hotel Santon. A total 80 of scientists, experts of veterinary administration or lawyers participated in the meeting. Most papers were devoted to welfare and laboratory animal protection; other presentations reported new experimental methods or data from experiments both in animals or new alternative models. The amended regulations and laws at the care of laboratory animals were presented, too.

MAINTAINING MODEL PARASITES IN LABORATORY ANIMALS – TOWARDS MEETING THE 3R'S REQUIREMENTS

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The recent European legislation regulating animal experiments (i.e. Directive 2010/63/EU) and in the Slovak Republic also Act. No. 377/2012 for the first time embody the application of the principles of 3Rs (i.e. Replacement, Reduction and Refinement) as defined by Russel and Burch in 1959. In addition to laboratory animals' housing conditions, the legislation sets new requirements to the experimental designs applied by the researchers to protect the animal welfare. In the Animal Facility of Institute of Parasitology SAS, several model parasites are maintained for scientific and educational purposes – European *Trichinella* reference strains in laboratory mice, *Echinococcus multilocularis* larvocysts in gerbils, and *Mesocestoides corti* larval stages in mice. Since 2013, the objective evaluation of score has been established for experimental animals, based on the classical General distress scoring sheet by Morton and Griffiths (1985). Using the scheme, appearance, food and water intake, clinical signs, natural and provoked behavior are monitored and scored on regular basis by facility staff. If the overall score reaches 10 or more for three consecutive days, the animal is found to be deteriorating, the experimental end point has been reached and the euthanasia is actively considered. In maintaining the *Trichinella* parasites, also the results of dose-response experiment were implemented to minimize the number of animals needed and to maximize the number of larvae obtained for individual strains. The use of such system led to improvement of animal care and prevention of severe distress and death and allowed to reduce the number of animals needed.

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RECOMBINANT CONGENIC STRAINS OF MICE – FIRST AND THE ONLY GENETIC MODEL FOR THE STUDY OF SUSCEPTIBILITY TO THE PARASITE *LEISHMANIA TROPICA*

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Leishmaniasis is endemic in 98 countries on 5 continents, causing 20,000 to 40,000 deaths per year (Alvar *et al.* 2012). The disease is caused by intracellular protozoan parasites of *Leishmania* species and transmitted by phlebotomine sand flies. Infection represents an important global health problem, as no safe and effective vaccine currently exists against any form of human leishmaniasis, and the treatment is hampered by serious side effects (Kobets *et al.* 2012a). In humans, *L. tropica* causes cutaneous lesions in the site of the insect bite, but the parasites can also penetrate to internal organs. This involvement of *L. tropica* in visceral human leishmaniasis has been recognized only recently. Animal models are a powerful tool for elucidation of host-parasite interactions (Lipoldová and Demant 2006), but the literature about the use of inbred animal models for *L. tropica* studies is scant. Thus, the aim of our research was to establish a defined model for analysis of disease caused by *L. tropica*. To achieve this, we adapted SNB medium for obtaining highly infective *Leishmania* promastigotes (Grekov *et al.* 2011), developed highly sensitive technique for detection and quantification of *Leishmania* parasites (Kobets *et al.* 2010) and used a robust genetic system – the recombinant congenic (RC) strains of mice, developed for analysis of multigenically controlled biological processes (Demant and Hart 1986). We studied susceptibility to *L. tropica* using BALB/c-c-STS/A (CcS/Dem) series of RC strains. Each CcS/Dem strain carries a unique set of 12.5 % of the genes from the donor parental strain STS, and 87.5 % of the genes from the second

parental strain, the background strain BALB/c. Mice (both males and females) of parental strains BALB/c and STS and RC strains CcS-3, CcS-5, CcS-11, CcS-12, CcS-16, CcS-18, and CcS-20 were infected with *L. tropica* and skin lesions, cytokine and chemokine levels in serum, and parasite numbers in organs were measured. Strains STS and CcS-5 were resistant to skin lesions development, females of the strains BALB/c, CcS-11, CcS-16 and CcS-20 were relatively susceptible to the infection and developed skin lesions; the largest lesions were observed in CcS-16. In some strains parasites visceralized and were detected in spleen and liver. The majority of *L. tropica*-infected strains exhibited increased levels of chemokines CCL2, CCL3 and CCL5. CcS-16 females exhibited a unique systemic chemokine reaction, characterized by additional transient early peaks of CCL3 and CCL5, which were not present in CcS-16 males nor in any other strain (Kobets *et al.* 2012b). The most susceptible strain CcS-16 was therefore selected for further studies. In female F₂ hybrids between BALB/c and CcS-16 we detected and mapped eight gene-loci, *Ltr1-8* (*Leishmania tropica* response 1-8) that control various manifestations of disease: skin lesions, splenomegaly, hepatomegaly, parasite numbers in spleen, liver, and inguinal lymph nodes, and serum level of CCL3, CCL5, and CCL7 after *L. tropica* infection. The most precise mapping (4.07 Mb) was achieved for *Ltr1* (chr.2), which controls parasite numbers in lymph nodes. These loci are functionally heterogeneous – each influences a different set of responses to the pathogen. *Ltr3* exhibits the recently discovered phenomenon of trans-generational parental effect on parasite numbers in spleen. Five loci co-localize with the previously described loci that control susceptibility to *L. major* (Badalová *et al.* 2002, Havelková *et al.* 2006, Vladimirov *et al.* 2003), three are species-specific. *Ltr2* co-localizes not only with *Lmr14* (*Leishmania major* response 14), but also with *Ir2* influencing susceptibility to *L. donovani* and might therefore carry a common gene controlling susceptibility to leishmaniasis. This is the first identification of genetic loci controlling susceptibility to *L. tropica* infection in any species (Sohrabi *et al.* 2013).

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SOCIAL BEHAVIOR OF GIBBONS (*HYLOBATIDAE*)

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Gibbons (*Hylobatidae*) are arboreal primates of a South-East Asian tropical forest, included to superfamily apes (*Hominoidea*). Opinions of their systematic distribution are different, some authors recognize even 16 species, the lowest number by other authors is 9 species, but with a lot of subspecies. Nutrient in the wild is made by mostly ripe fruits but part of their diet can be also young leaves and browse. To a small extent gibbons regularly eat animal diet, for example insect and bird eggs. The species most kept in captivity is White-handed gibbon (*Hylobates lar*). A longevity record of 60 years spent in captivity holds a Mueller's gibbon (*Hylobates muelleri*). The second part is concerned to social behavior. At the beginning are mentioned connections between ethology, ethoecology and sociobiology, all about animal behavior. After this follows chapter of definition of social behavior. The last and most important part of this paper is directed to social behavior of gibbons, specifically about gibbons social structure, parental behavior, territorial behavior, communication (especially acoustic and tactile), dietary behavior, agonistic and aggressive behavior, and gibbons behavior during the day. Gibbons live in monogamous social structure, more precisely is it monogamy for a whole life or permanent monogamy, but last researches shows that this structure is not reproductively isolated. Extra-pair copulations may be explained as an effort to breed with a partner of superior quality to the current mate and / or may be part of a strategy to forestall infanticide. Gibbons family usually includes father, mother and the young to a certain age. There are no more than four young ones in different age. Gibbons indicate their territories by a singing. Acoustic communication is very important for them. Vocalization is different by every species; females usually have more complex melodies. The biggest threat for gibbons is losing of their habitat and illegal hunting. The Hainan gibbon (*Nomascus hainanus*) is probably the most endangered primate in the world, it is known only 20 individuals of this species, and just three of them are breeding females.

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ENVIRONMENTAL ENRICHMENT POSSIBILITIES IN LABORATORY FERRETS

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The ferrets are intelligent, perceptive and lively animals, it is therefore necessary to enrich their environment, as well as to inform the public of the way of breeding, due to the fact that the ferrets are bred more and more as domestic animals. That is why the following part of the work deals with the breeding itself, which is divided into the breeding in laboratory and domestic conditions. The work describes trends of breeding from the past century up to the present and refers to the most common mistakes in order to correct them. It concerns with the whole scale of issues, from food enrichment to toys and accessories for ferrets. The final part of the work focuses on castration, which substantially interferes in the life of animals and it is often practiced in case of breeding ferrets as well. Regarding the discovered findings, it is apparent that the living conditions for breeding ferrets are incomparably better at present, and that the attention paid to this subject matter is increasingly bigger. On the other hand, there are still problems related to the breeding, which are caused either by badly acquired methods or by unprofessional literature dedicated to ferrets.

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NATIONAL INSTITUTE OF PUBLIC HEALTH – CONTACT POINT AND REFERENCE LABORATORY FOR ALTERNATIVE METHODS ACCORDING TO DIRECTIVE 2010/63/EU

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The Czech Republic as a member state of the EU has implemented the Directive 2010/63/EU on the protection of animals used for scientific purposes into the Act No. 359/2012, amending Act No. 246/1992, on the protection of animals against cruelty. The new directive and amended act include the protection of animals used for scientific purposes and for toxicological testing. The aim of the new directive is the reduction of animals in tests, refinement limiting their suffering and replacement of animal testing by alternative *in vitro* methods utilizing cells and tissues of human origin. According to the Directive 2010/63/EU, the member states are obliged to ensure support of alternative methods and dissemination of information. They should nominate a single point of contact to provide advice on the regulatory relevance and suitability of alternative approaches respecting the 3Rs, which becomes a member of PARERE network. The member states should also nominate to the European Commission suitable specialized and qualified laboratories to carry out validation studies. The Ministry of Agriculture has nominated as the contact point and specialized laboratory the National Institute of Public Health (NIPH) in Prague, namely the National Reference Laboratory for Experimental Immunotoxicology at the Centre of Toxicology and Health Safety, headed by Dagmar Jírová, M.D., Ph.D. Following a selection procedure, the nominated specialized laboratory at NIPH has been appointed into the network of 26 national reference laboratories (NETVAL) as the only representative from Central and Eastern Europe. The appointment is based on internationally recognized scientific, personal and laboratory qualification of the facility in the field of development and implementation of alternative methods in toxicological practice, participation in a number of national and international scientific projects and personal reputation of team members, who were appointed as members of EURL-ECVAM Scientific Advisory Committee, or members of working groups and committees of the OECD and European Chemical Agency. The presented poster summarizes the range of activities of the contact point and reference laboratory, including a list of implemented alternative methods and examples of scientific results (e.g. skin penetration of TiO₂ nanoparticles). The prestige of the NIPH laboratory is highlighted by hosting the 9th World Congress on Alternatives to Animal Experiments in Prague, 2014 (<http://www.wc9prague.org>). This congress represents an occasion with worldwide impact on rational use of animals for scientific purposes and effective implementation of validated alternative methods into testing strategies.

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SYSTEMIC AND LOCAL TOXICITY OF API INTERMEDIATES IN MICE AND RATS

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Toxicological research and testing is necessary part for determination how much of a test substance is safe and how much is hazardous to human health. Although there are many alternative methods *in vitro*, laboratory animals still play the most important role in toxicological studies. The aim of the work was to evaluate the acute toxicity and sensitizing potential of API intermediates C8 and C17. Test results of acute oral and dermal toxicity in Wistar rats indicated low toxicity of the both items. Within the set of tests LD50 was more than 2000 mg/kg except for C8 acute oral toxicity – 1750 mg/kg. Results of Local Lymph Nodes Assay (LLNA) in mice CBA/Ca confirmed sensitizing potential of the both intermediates. Local side effect in exposed areas was observed after three days application. Histopathologic examination demonstrated necrosis and inflammatory infiltration in injured areas in the highest concentration of test substances.

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THE ROLE OF IMMUNE CELLS IN PROGRESSION AND SPONTANEOUS REGRESSION OF THE LEWIS RAT SARCOMA MODEL

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Spontaneous regression (SR) of tumors is a fascinating phenomenon observed rarely in oncological patients. Mechanisms responsible for SR are not well known. Participation of immune system in SR was suggested. Using a suitable rat model with progressing and spontaneously regressing sarcoma we detected the role of various immune cell types in these processes. The R5-28-2 rat sarcoma cells (C4 clone) at dose 5x10⁵ were inoculated subcutaneously to 21 female Lewis rats. Four rats without inoculation served as the control group. Basic hematological parameters of peripheral blood (determined by the Vet ABC analyzer), blood smears (stained with May-Grünwald and Giemsa-Romanovski solutions) and tumor size were monitored once a week. Various types of immune cells were detected on tumor cryosections by immunofluorescence with anti-rat CD antibodies. Tumor progression (TP) and SR was observed in 29 % and 38 % of inoculated rats, respectively. The remaining 33 % of experimental animals did not develop any tumor. Hematological parameters (number of red blood cells and platelets, hemoglobin concentration, hematocrit) in peripheral blood were not affected or they slightly decreased in rats with TP whereas they increased gradually during experiment in the control group (p<0.05). The number of platelets rapidly increased in rats with SR compared to TP and controls (p<0.05). In rats with SR, the values of these parameters were decreased until the tumor totally disappeared (i.e. around the 45th day after inoculation) and thereafter they were aligned to normal levels. Number of white blood cells (WBC) increased significantly (p<0.05) in both TP and SR. This change was caused mainly by the expanded population of granulocytes as observed in blood smears and cryosections. After total tumor absorption in SR, this cell population went back to normal value. Immunohistochemical analyses also revealed in TP a low number of NK cells (CD 161 positive) that were irregularly dispersed in tumor sections. On the contrary, very dense infiltration of NK cells was found in SR. T helper cells (CD 4 positive) and cytotoxic T lymphocytes (CD 8 positive) showed decreased number in SR and TP in comparison with controls (p<0.05). Group of rats that did not develop any tumor showed similar values of studied parameters as the control group. The obtained results in the rat sarcoma model suggest that NK cells play a key role in SR. The expanded population of granulocytes may shift tumor development to TP or SR depending on the cytokine milieu.

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USE OF NON-INVASIVE ELECTROGASTROGRAPHY FOR STUDIES OF THE EFFECT OF XENOBIOTICS (MODEL DRUG: ATROPINE) ON GASTRIC MYOELECTRIC ACTIVITY IN EXPERIMENTAL PIGS

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Surface electrogastrography (EGG) is a non-invasive method for clinical assessment of gastric myoelectrical activity. Our group has demonstrated in previous studies that EGG is also reliable and feasible in experimental pigs. Porcine EGG is fully comparable with that recorded in healthy humans. The purpose of this study was to evaluate

the effect of atropine, as a representative of anticholinergic agents, on gastric myoelectrical activity in experimental pigs. Six young mature female pigs entered the study three times in a random order. The baseline EGG recording lasted 20 min, followed by a 105-min EGG trial recording. Intramuscular atropine 1.5 mg (part 1), 3.0 mg (part 2) and 4.5 mg (part 3) was administered after the baseline EGG. Surface cutaneous EGG was recorded under general anesthesia. Running spectral analysis (based on Fourier transform) and power analysis were used for the EGG evaluation. Pulse oximetry was used to secure the experiment and heart rate served as a marker of the myocardial effect of atropine. Although statistically significant in a few time intervals after atropine administration, all changes in the dominant frequency were within the normal range in all parts. In the power analysis, the areas of amplitudes increased from basal median values ($296.5 \mu V^2$) to median 335 (part 1) and median 423 (part 2) after 15 min ($p=0.029$). After that period, the areas of amplitudes decreased significantly to median 178 (part 1) and median 316 (part 2) after 60 min ($p=0.020$). Finally, they increased to median 226 (part 1) and median 1389 μV^2 (part 2) after 105 min ($p<0.001$). In part 3, amplitudes gradually decreased from basal values to median 114 μV^2 after 105 min ($p<0.001$). Changes in gastric myoelectrical activity after different doses of atropine were relatively small in the dominant frequency. Significant dose-dependent changes in the areas of amplitudes were found in the power analysis. The heart rate confirmed the expected changes after atropine administration.

DETECTION OF THE INFLUENCE OF THE CYSTIC FORMS OF *BORRELIA BURGDORFERI* SENSU LATO TO THE EXPERIMENTAL MICE BALB/C UNIT

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The ability of the bacteria *Borrelia burgdorferi* sensu lato viable spiral form to generate cystic form has been published for many years. But the impact of the cystic forms on the immune system of the infected individuals is not explored enough so far, nor the role of this form in the process of Lyme disease is yet understood. The aim of our study was to assess the effect of the cystic forms *Borrelia burgdorferi* sensu lato on the immune response in treated experimental subjects *Mus musculus* (BALB/c). Cystic forms of different age (3, 10 and 17 days) were prepared and injected intraperitoneally into the tested mice. The effect of the cystic forms on the immune response was monitored during a five week period using the detection of specific IgG and IgM antibodies in a serum of the infected individuals by ELISA (Enzyme-linked immunosorbent assay) method. No influence of the cystic forms to the level of specific antibodies was shown during the study, by comparison of the level of antibodies generated by a control group of mice immunized by *Borrelia* motile viable cells and a group of mice treated by *Borrelia* somnified cells, which generated antibodies. The method of cultivation of the cystic forms in BSKII (Barbour-Stoenner-Kelly) medium demonstrated the ability of these forms to reconvert back into the viable spiral forms, such ability depends on the age of the cysts. With increasing age, the ability of the cysts to return to the spiral form declines. Similarly a patented method Dualdur® was carried out, where motile forms of bacteria were detected in the blood of the experimental animals. The difficulty to detect the cystic forms in samples either by ELISA or other molecular and microbiological methods hinders the diagnosis and treatment of Lyme disease. From this perspective, it is obvious that a close attention towards etiology of the cystic forms of *Borrelia burgdorferi* sensu lato is needed for further research.

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TESTING OF SUBSTANCES WITH POTENTIAL EFFECT AGAINST *MYCOBACTERIUM TUBERCULOSIS*

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Laboratory mice will be infected with *Mycobacterium tuberculosis* intranasally (i.n.). This administration allows safe application of

infectious organisms at accurately defined quantity of microorganism cells. Infectious process, induced by such way, maximally simulate natural transmission of infection. Subsequently, therapy with selected substances with potential effect against *Mycobacterium tuberculosis* will be established. Application of tested drugs will be performed parenterally, or eventually by another route. Stabling conditions of experimental animals (temperature, humidity, biorhythm compliance etc.) will be observed continuously. All tests will be performed according the law No. 246/1992 Sb. and related regulations. Visual manifestation of proceeded infection and pertinent changes of body weight in tested animals will be observed during the experiment. Tissue samples from spleen and lungs for determination of CFU (colony forming units) will be taken after euthanasia.

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BLOOD COLLECTION TECHNIQUE; IMPACT ON ANIMAL HEALTH

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The aim of the lecture was to present usage of one of the three principles "R", it is referment procedure of blood collection in experimental animals. The blood collections are usually need for diagnosing diseases, the pharmacology, the biochemistry examination and research. It is necessary for maintaining the good health of animals in respect for the total blood volume, which is from 5.5 % to 7 % of their body weight. Overstepping these limits can happen especially during multiple blood collections. The volume over 15 % of the total blood volume cause false hematological, biochemical results and increased concentration of stress related hormones. The clinical symptoms of hypovolemic shock are observed during the loss of blood over 30 % of the total blood volume and blood loss over 40 % caused mortality. It was described the most common techniques of blood collection in experimental animals and it was evaluated its benefit and risk. Blood collections in large animals (rabbits, poultry, swine, sheep, goats etc.) does not cause problems in ear veins, jugular veins and other superficial veins. The blood collection from superficial veins in mice and rats is commonly done on tail veins, but it is possible to take only a small volume of blood, which can be contaminated by pieces of skin. Collection of larger blood volume in mice, rats, guinea pigs etc. is still the preferred technique from retro-orbital sinus, but the disturbance causes bleeding from collection site and has a negative impact on the state of animal health. Therefore, the technique from cranial vena cava was described instead from retro-orbital sinus and cardiac punctures in rats and guinea pigs, respectively. Animal under short-term injected anesthesia was placed in dorsal recumbency and the needle was inserted at a 30° angle to the cranial at the first rib at the injection site, about 5 mm lateral to the manubrium in direction of opposite iliac joint. After skin penetration, negative pressure was induced in the syringe and the needle was inserted a further 0.5 upward 1 cm until blood began to flow. After blood collection, the needle was withdrawn and digital pressure was applied at the site injection for 30 s. In the lecture, the technique was described of blood collection from cranial vena cava in rats and guinea pigs instead retro-orbital sinus in rats and cardiac puncture in guinea pigs. This technique with respect to the animal health is not only safety, but also gentle and gives valid results in trials on experimental animals and is better in regards to the animal welfare.

ADVERSE EFFECTS OF RARE EARTH ELEMENTS (REEs) DETERMINED BY ALTERNATIVE METHODS

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Rare earth elements (REEs) were on the edge of scientific interest until recently, as it turned out that the effect of REEs on living organisms is much higher than expected. They are widely used in a variety of products, automobile industry, glass industry, agriculture, medicine and electronics (magnets and batteries). The predominant supplier is China with approximately 80 % of REEs mineral reserve in the world. During the last 20 years these elements were widely used in Chinese agriculture

as fertilizers and as animal growth promoters. However, the scientific literature in this field is fragmented and research of REEs in “western conditions” is still developing. According to the latest studies, at suitable concentrations REEs promote physiological functions in plants, stimulate growth and increase chlorophyll content. Recently REEs are of interest in animal production as an alternative growth promoter. Since the use of antibiotics as growth promoters has been absolutely restricted in the European Union, new possibilities are searched. It is already known that REEs increase body weight gain, milk and egg production, though their concentration in final products is very low or below the limit of detection. Since lanthanum carbonate is a novel treatment for hyperphosphatemia in dialysis patients, long-term studies were performed in man. Up to 3 g of elemental lanthanum per day and person for up to 4 years were well tolerated without any toxic effects. However, the information on adverse effects and toxicity is scarce, and the presented study aimed to extend the knowledge of their toxicological profile. The tested compounds included 8 REEs salts. The toxicity tests comprised the 3T3 Balb/c NRU cytotoxicity test for calculation of LD₅₀ on the basis of the concentration which led to a 50 % reduction in cell growth (IC₅₀), and the Tubifex tubifex (T.t.) express test (3 min) for acute toxicity, measuring movement inhibition of the oligochaete worms (EC₅₀). Mutagenic effects were determined using the bacteria reverse mutation assay (Ames test) on 5 Salmonella typhimurium strains with and without metabolic activation. The acute toxicity levels calculated from the 3T3 results were in the range of 962-1123 mg/kg indicating toxicity for mammals. The EC₅₀(T.t.) values were comparable with those of, e.g., BaCl₂ or CdCl₂, classified as toxic and aquatic toxic compounds. Thus, the EC₅₀(T.t.) indicates the hazard of rare earth elements salts for aqueous environment as well. The Ames test provided negative results for all the tested REEs salts. Further testing aimed at eye/skin irritation, genotoxicity, skin penetration and sensitization is planned in the future.

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