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Contents

Working Party Report

ESLAV/ECLAM/LAVA/EVERI recommendations for the roles, responsibilities and training of the laboratory animal veterinarian and the designated veterinarian under Directive 2010/63/EU
GM Poirier, C Bergmann, DG Denais-Lalieve, IA Duntas, N Dudoignon, H Ehall, JM Fentener van Vlissingen, M Fornasier, R Kalman, A Hansen, S Schueller, P Vergara, R Weilenmann, J Wilson and A-D Degryse

Original Articles

Safety studies of post-surgical buprenorphine therapy for mice
KA Traul, JB Romero, C Brayton, L DeTolla, N Forbes-McBean, MS Halquist, HT Karnes, R Sarabia-Estrada, MJ Tomlinson, BM Tyler, X Ye, P Zadnik and M Guarnieri

The effect of midazolam on the recovery quality, recovery time and the minimum alveolar concentration for extubation in the isoflurane-anesthetized pig
SA Kleine, JE Quandt, EH Hofmeister and J Peroni

Using the mouse grimace scale to assess pain associated with routine ear notching and the effect of analgesia in laboratory mice
AL Miller and MC Leach

Assessment and refinement of intra-bone marrow transplantation in mice
U Pfeiffenberger, T Yau, D Fink, A Tichy, R Palme, M Egerbacher and T Rülicke

Comparison of haematopoietic stem cell engraftment through the retro-orbital venous sinus and the lateral vein: alternative routes for bone marrow transplantation in mice
D Leon-Rico, M Fernández-García, M Aldea, R Sánchez, R Sánchez, M Peces-Barba, J Martinez-Palacio, RM Yáñez and E Almarza

Hemorheology in experimental research: is it necessary to consider blood fluidity differences in the laboratory rat?
U Windberger, K Spurny, A Graf and H Thomae

Coil optimization for low-field MRI: a dedicated process for small animal preclinical studies
T Feuillet, M-J Seurin, O Leveneur, E Viguier and O Beuf

Short Reports

Non-invasive 3D time-of-flight imaging technique for tumour volume assessment in subcutaneous models
JAD San Martin, P Worthington and JWT Yates
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Working Party Report

ESLAV/ECLAM/LAVA/EVERI recommendations for the roles, responsibilities and training of the laboratory animal veterinarian and the designated veterinarian under Directive 2010/63/EU

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Abstract
Directive 2010/63/EU was adopted in September 2010 by the European Parliament and Council, and became effective in January 2013. It replaces Directive 86/609/EEC and introduces new requirements for the protection of animals used for scientific purposes. In particular, it requires that establishments that breed, supply or use laboratory animals have a designated veterinarian (DV) with expertise in laboratory animal medicine, or a suitably qualified expert where more appropriate, charged with advisory duties in relation to the well-being and treatment of the animals. This paper is a report of an ESLAV/ECLAM/LAVA/EVERI working group that provides professional guidance on the role and postgraduate training of laboratory animal veterinarians (LAVs), who may be working as DVs under Directive 2010/63/EU. It is also aimed at advising employers, regulators and other persons working under the Directive on the role of the DV. The role and responsibilities of the DV include the development, implementation and continuing review of an adequate programme for veterinary care at establishments breeding and/or using animals for scientific purposes. The programme should be tailored to the needs of the establishment and based on the Directive’s requirements, other regulations, and current guidelines in laboratory animal medicine. Postgraduate laboratory animal veterinary training should include a basic task-specific training module for DVs to complement veterinary competences from graduation, and continuing professional development on the basis of a gap analysis. A tiered approach to further training in laboratory animal veterinary medicine and science offers career development pathways that are mutually beneficial to LAVs and establishments.
Keywords
designated veterinarian, Directive 2010/63/EU, laboratory animal veterinarian, roles and responsibilities, well-being

Veterinarians are leading advocates for animal welfare and have the responsibility to ensure the health and welfare of all animals under their care. The replacement of Council Directive 86/609/EEC1 by Directive 2010/63/EU2 on the protection of animals used for scientific purposes provides a legal environment for veterinarians to continue to fulfill this responsibility in the field of animal research. Article 25 of Directive 2010/63/EU requires that ‘each breeder, supplier and user has a designated veterinarian with expertise in laboratory animal medicine, or a suitably qualified expert where more appropriate, charged with advisory duties in relation to the well-being and treatment of the animals’. The Directive therefore offers an opportunity for developing a level playing field in the management of the health and welfare of laboratory animals throughout Europe. In May 2013 the European Commission invited the European Society of Laboratory Animal Veterinarians (ESLAV, http://eslav-eclam.org/) and the European College of Laboratory Animal Medicine (ECLAM, http://eslav-eclam.org/), amongst other non-governmental organizations, to designate an expert to participate in the 3rd Meeting of the Expert Working Group (EWG) on Education and Training within the context of Directive 2010/63/EU and to prepare a thought-starter document on the following topics: (i) roles and responsibilities of the designated veterinarian (DV) (Article 25 of the Directive); (ii) training needs for the DV; (iii) training needs for veterinarians involved in project evaluation (Article 38 of the Directive); (iv) role of the veterinarians in the training of persons in Article 24 (1) (a–c) of the Directive. As for previous meetings of this EWG, ESLAV and ECLAM accepted this invitation and invited the Laboratory Animal Veterinary Association (LAVA, http://www.lava.uk.net/) in the preparation of the document in view of LAVA’s long-standing experience with the concept and responsibilities of the Named Veterinary Surgeon (NVS) under the Animals (Scientific Procedures) Act 19863 in the UK. The Board of the Association for European Veterinarians in Education, Research and Industry (EVERI, http://www.fve.org/about_fve/sections/EVERI.php) was involved at a later stage in the writing of the paper and endorsed it.

This paper is based on the interpretation of the Directive by and presents the views of experienced laboratory animal veterinarians (LAVs). The aims are (i) to provide professional guidance on the role and responsibilities of DVs and LAVs such as proposed by the endorsed document on the ‘Development of a Common Education and Training Framework to Fulfil the Requirements under the Directive’; (ii) to highlight the need for postgraduate veterinary training in this field at the minimum DV level as agreed by EU Member States, and mid-tier level as anticipated by Veterinary Continuing Education in Europe (VetCEE), a recent European accreditation scheme; and (iii) to discuss postgraduate training opportunities for DVs and LAVs.

Background
ESLAV, ECLAM, LAVA and EVERI recognize that the use of animals is currently still necessary for scientific progress and for the discovery of new medicines that will ultimately benefit humans and animals. These groups also recognize that veterinarians have professional, legal and ethical obligations to protect animal health and welfare and share the mission of ensuring high standards for the care and use of laboratory animals, through the continuing education, training, and postgraduate qualification of LAVs. ESLAV was founded in 1996, ECLAM in 2000, and both organizations are well recognized within and outside Europe. ESLAV and ECLAM have a strong membership of veterinarians working in laboratory animal medicine and science (LAMS) across European countries and beyond (356 members in 33 countries, of which 81 are ECLAM Diplomates). LAVA was founded in 1963 and now has 161 members. Based on the membership of these organizations and the existence of similar professional organizations in EU Member States, it is estimated that more than 1000 veterinarians are employed in the field of laboratory animal medicine and science in Europe fulfilling one or more of the responsibilities stated in Directive 2010/63/EU. EVERI, one of the sections of the Federation of Veterinarians in Europe (FVE, http://www.fve.org), is an umbrella organization of National and European associations of veterinarians employed in the sectors of education, research and/or industry, that was founded in 2005.

LAVs have been providing advice on animal health and welfare to establishments involved in the breeding or use of animals for scientific purposes for many years in Europe and in other parts of the world.
In 2012 ESLAV and ECLAM conducted an online survey on the role, education and training of LAVs and their expectations with regard to the impact of changes associated with the implementation of Directive 2010/63/EU. A total of 123 LAVs participated in the survey, out of which 30% were ECLAM Diplomates, and 50% were holders of another Diploma (e.g. PhD, or Diploma in Laboratory Animal Science). More than 80% of the responders were working in a full-time position.

Responses indicated that LAVs are currently involved in a broad range of activities related to the functions of ethical committees, facility management, health monitoring, research projects, occupational health and safety, compliance with legislation and the training of persons using animals for experimental purposes (Table 1), and expected continued involvement under the new Directive (Table 2). LAVs also provide scientific and ethical advice on experimental projects with regard to the application of the 3R principles (replacement, reduction and refinement) that were originally proposed by Russell and Burch and are now embedded in the Directive.

**Veterinary roles, tasks and responsibilities under Directive 2010/63/EU**

**Veterinary tasks required under the Directive**

Article 25 of Directive 2010/63/EU requires that ‘each breeder, supplier and user has a designated veterinarian with expertise in laboratory animal medicine, or a suitably qualified expert where more appropriate, charged with advisory duties in relation to the well-being and treatment of the animals’. The list of veterinary roles and responsibilities that are specifically mentioned in the Directive is shown in Table 3. Of importance, establishments have the obligation to ensure that veterinary care is available at all times. It is also worth noting that amongst the veterinary responsibilities listed in the Directive, the only one that is strictly referred to the DV is the provision of veterinary input to the animal welfare body (AWB). This allows a degree of flexibility in the way veterinary services could be organized at establishments. For instance, depending on the size and complexity of the establishment, the roles of the DV may be played by one or more LAVs. In the latter case, it would be beneficial for the veterinarian(s) charged with DV responsibilities to have oversight of all veterinary matters related to animal health and welfare.

The Directive offers limited guidance on (i) veterinary care programme; (ii) veterinary input to the AWB; (iii) the role of the veterinarian in training others; (iv) the interface between the Directive and other veterinary regulations; and (v) the importance of maintaining effective communications with other persons working in the establishment. These aspects are developed in the following sections of this paper.

**Provision of an adequate veterinary care programme**

It is the professional view of the authors that the veterinary services and tasks that are stated in the Directive should form the basis of a comprehensive veterinary care programme that is tailored to the needs, complexity and purpose of the establishment. The concept of a programme of veterinary care for laboratory animals has been described in the 2008 report of the FELASA/
The veterinary care programme should include all veterinary services and advice that are relevant to the health and welfare of animals that are transported to or from an establishment, received, maintained in acclimatization or in quarantine, bred or used at the establishment. The main elements of the programme of veterinary care are described in Table 4. They form the basis for the provision of advice, recommendations, and guidelines on husbandry, ethical, scientific, technical and safety practices, in addition to the conduct of clinical veterinary medicine and surgery. It is essential that the programme of veterinary care is regularly reviewed and updated according to ongoing progress in these areas.

**Input to the AWB**

The Directive does not require a veterinarian to be a full member of the AWB, but requires that the AWB seeks veterinary input from the DV or the expert referred to in Article 25 (Article 26). Expert advice in laboratory animal medicine is relevant and important to the five main functions assigned to the AWB by the Directive: (i) advice on animal welfare; (ii) advice on the principles of the 3Rs; (iii) establishment and review of procedures that may impact animal welfare; (iv) continuous monitoring of projects and advice on opportunities for application of the 3Rs; and (v) advice on re-homing of animals. To fulfil this role the DV needs to have sufficient awareness and understanding of the local practices, and needs to be able to contribute to discussions taking place at the AWB. It is therefore the view of this working group that the DV should be a full member of the AWB.

**Involvement in project evaluation**

The Directive requires that projects are evaluated and authorized by the relevant National competent authority (Articles 36, 37 and 38). In some establishments a local ethical review process may be established to enable the evaluation and the refinement of project proposals prior to their evaluation by the competent authority.
authority. In such circumstances, the DV and/or an LAV working with the DV may be requested to contribute to the review of project proposals. Veterinary input in the ethical review of projects brings critical information and recommendations on the design of protocols (Article 38.3b) that support the application of the 3Rs. Advice include considerations for the choice of species or animals, the choice of animal model, the relevance of a procedure for the intended purpose, the reduction of bias in study results, the refinement of procedures and/or animal care in order to minimize its severity, the classification of the severity of the procedure, and the establishment of humane endpoints.

### Training, supervision and assessment of competences of other persons

Due to the breadth of skills and knowledge required to ensure adequate animal care and use in scientific procedures, the training of personnel participating in these activities often follows a multidisciplinary approach, in which experts are invited to provide training in their focused area of expertise. In this context, veterinarians have a professional responsibility of ensuring that activities normally considered as acts of veterinary practice under National laws are performed in accordance with standards accepted by the veterinary profession, whether they are performed by veterinarians or by other persons allowed to perform such activities under Directive 2010/63/EU. Veterinary have a solid base of knowledge and expertise in comparative pathology, diagnosis, prognosis, disease prevention and treatment, anaesthesia and surgery, pain recognition and control, breeding control, and euthanasia that is relevant to laboratory animals. They are therefore uniquely qualified to provide training, assessment and supervision on what is considered to be veterinary interventions for scientific procedures. Consequently, it is strongly recommended that they are included in the provision of training and supervision to others.

### Interface between the Directive and veterinary regulations

A complex veterinary legal and regulatory framework exists at National and EU levels. This forms an important interface with the Directive 2010/63/EU which requires special attention. Laws and codes of practice...
acknowledge the specific training and expertise needed to perform acts of veterinary practice, including the provision of anesthesia, surgery and the control of the use of medicines. Directive 2010/63/EU permits other suitably qualified persons to provide advice in relation to animal health and welfare. The authors recommend that the decision process aimed to nominate another suitably qualified person involves consultation with a veterinarian with sufficient knowledge and training in laboratory animal medicine. In addition, when acts of veterinary practice have to be executed, which are assigned to licensed veterinarians in other regulations, such as a surgical procedure to relieve an animal from pain not related to the experimental protocol, those should be performed by a licensed veterinarian or under his/her responsibility and direct supervision. Similarly the acquisition and use of controlled substances and veterinary medicines should be compliant with European and National veterinary medicines laws and regulations. Finally, the knowledge that is necessary to fulfil obligations to report public health hazards to the authorities should also be taken into account.

Veterinary services should comply with specific National and EU legal requirements aimed at protecting the health and welfare of both animals and humans. These regulations apply to a wide range of veterinary services in relation to exotic animals, animal by-products, hygiene, animal transport, and import and export of animals between establishments and countries. Veterinary advice may also be sought in the context of the Convention on International Trade in Endangered Species (CITES, www.cites.org).

**Communication lines involving the LAV**

Although the role of the DV is advisory under the Directive, in practical circumstances it is important that veterinary advice is carefully considered. For this to happen, the DV will need to have sufficient competence, to gain respect from colleagues and be provided with adequate support and authority by the establishment.

The DV and additional LAVs need to interact with a variety of professionals within and outside the establishment. The DV has a statutory responsibility towards the representative of the local establishment in addition to other government and professional bodies. He/She also frequently interacts with persons that are responsible for projects or those that work with animals in order to provide advice on matters related to health and welfare of protected animals. To ensure that advice is given when needed, clear communication lines should be established between the DV and the person(s) responsible for overseeing the welfare and care of the animals, and a clear process indicating when and how the DV should be contacted when health or welfare issues occur should be agreed (Article 24.1). The DV should also build and maintain constructive relationships with scientists in order to develop mutual understanding and respect.

In order to keep everyone abreast with developments in the 3Rs, the DV should actively engage with and contribute to the activities of the AWB, thereby enabling the establishment to be updated.

Communication lines should therefore be established between the DV and the persons and group indicated below:

- The person responsible for ensuring compliance with the provisions of the Directive (Article 20.2);
- The person(s) responsible for overseeing the welfare and care of the animals in the establishment (Article 24.1);
- The AWB (Articles 26, 27);
- The personnel working with animals (Article 23);
- Scientists responsible for projects (Article 24.2);
- The DV’s line management.

The DV should also be supported in maintaining links with laboratory animal veterinary associations, other professional networks and associations in the field of laboratory animal medicine and science such as the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC, http://www.aaalac.org/), International or National laboratory animal science associations, as well as diagnostic and other laboratories. The DV should develop and maintain effective communications with the competent authority.

**Employment modalities for the DV**

The employment remit of the DV should include responding to the needs of the establishment based on its size, complexity and purpose. Consultancy-based or part-time employment may be suitable at some establishments. In such cases, the DV should ensure that appropriate veterinary care is available at all times, and routine visits to the establishment should be made at sufficient intervals for the health and welfare of the animals to be effectively monitored. The provision of guidance on an adequate animal care and use programme may be shared by several LAVs depending on the size, the complexity, the species bred or used, and the purpose of the establishment. In such cases it is recommended that each DV has overall responsibility for, and oversight of, the veterinary services provided.
Competence requirements for the DV

Mobility of veterinarians in Europe

Through their undergraduate training, veterinarians gain a strong basis in animal welfare and acquire extensive knowledge in the prevention, diagnosis and intervention concerning animals’ health and care as ‘day-one competences’ upon graduation. Training requirements of veterinarians have been harmonized across Europe in order to facilitate the free movement and mobility of veterinarians in Europe. Directive 2005/36/EC on the recognition of professional qualifications lays out minimum requirements for study programmes and the minimum training period leading to the qualification of Veterinary Surgeons (Article 38,3 and amendments). It guarantees the mutual recognition of professional qualifications between Member States by requiring them to consider the qualifications acquired elsewhere in the Community to allow access to regulated professions. The European Association of Establishments for Veterinary Education (EAEVE, http://www.eaeve.org) assures the quality of veterinary establishments by evaluating, promoting and further developing the quality and the standard of establishments for veterinary medical education and their teaching within the EU.

When designing training frameworks for DVs, consideration should be given to facilitate the mobility of DVs in Europe.

As shown in Table 5, minimum requirements for veterinary study programmes defined under Directive 2005/36/EC do not address the specific needs of the field of laboratory animal medicine and science. Therefore additional specialized postgraduate training is likely to be needed for most graduate veterinarians to effectively fulfil the responsibilities of the DV under the Directive. Changes in veterinary curricula may over time enhance ‘day-one competences’ in aspects of laboratory animal medicine and science, and thus modify the need for additional postgraduate training.

Current postgraduate training framework and opportunities to LAVs

A number of training and education opportunities leading to postgraduate qualifications for LAVs exist in Europe. These opportunities are recognized at the European and/or the National level, such as Masters, Certificates and Diplomas (Table 6). They are distinguished by their syllabus, the depth and breadth of the knowledge and skills that are gained (e.g. range of species, range of animal models), the duration of their training period, their degree of formality in the delivery

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**Table 5. Summary of the key training requirements for veterinarians stated in Article 38 of Directive 2005/36/EC on the recognition of professional qualifications.**

**Admission to training**

Admission to veterinary training shall be contingent upon possession of a diploma or certificate entitling the holder to enter, for the studies in question, university establishments or institutes of higher education recognized by a Member State to be of an equivalent level for the purpose of the relevant study.

**Duration of training**

Mutual recognition [between Member States] of veterinary surgeons is specified in Article 38 and Annex V, point 5.4.1 of this Directive. The training of veterinary surgeons shall comprise a total of at least five years of full-time theoretical and practical studies at a university or at a higher institute providing training is recognized as being of an equivalent level, or under the supervision of a university, covering at least the study programme referred to in Annex V, point 5.4.1.

**Competences acquired**

Training as a veterinary surgeon shall provide an assurance that the person in question has acquired adequate knowledge and skills in the following areas:

- knowledge of the sciences on which the activities of the veterinary surgeon are based;
- knowledge of the structure and functions of healthy animals, of their husbandry, reproduction and hygiene in general, as well as their feeding, including the technology involved in the manufacture and preservation of foods corresponding to their needs;
- knowledge of the behaviour and protection of animals;
- knowledge of the causes, nature, course, effects, diagnosis and treatment of the diseases of animals, whether considered individually or in groups, including a special knowledge of the diseases which may be transmitted to humans;
- knowledge of preventive medicine;
- knowledge of the hygiene and technology involved in the production, manufacture and putting into circulation of animal foodstuffs or foodstuffs of animal origin intended for human consumption.
of training, the quality of the experience gained (e.g. whether experience is gained under supervision), and the procedures for the verification of competences. Introductory training opportunities in laboratory animal medicine exist in Europe although they are not widely available (e.g. Royal College of Veterinary Surgeons (RCVS)-recognized NVS course in the UK, http://www.rcvs.org.uk). Other training courses that do not lead to a formal qualification but address specific topics of laboratory animal medicine and science are available online or through direct attendance.

Recommendations for the training and education of the DV

DVs should have a minimum set of recognized core competences in laboratory animal medicine and science enabling them to adequately perform their roles and fulfill their responsibilities under the Directive. The endorsed document on the ‘Development of a Common Education and Training Framework to Fulfil the Requirements under the Directive’ offers guidance on this topic. These competences should at least cover:

- European and National laws and regulations which are relevant to laboratory animals and to the role of the DV.
- Ethics, animal welfare and 3R considerations applicable to laboratory animals and the role of the DV; justifying the importance of good animal health and welfare and recognizing the relationship between health and welfare and scientific validity; identifying criteria used in making a harm–benefit analysis and being able to apply them; identifying the role of the DV in advising on choice of animal model and model refinement.
- Principles of the management of a programme of veterinary care, including husbandry, disease, surgery, anaesthesia and pain, and clinical assessment that are applicable to laboratory animals.
- Concepts in the design of scientific procedures and research projects; defining strategies for effective communication and explaining how these promote animal welfare and good science; seizing and reviewing opportunities to gather further veterinary information in laboratory animal medicine and science.

Training leading to minimum core competences for the DV should be taken prior to, or when this is not possible, within a year of taking up a role as a DV. A modular approach to this training would be beneficial because it would enable a degree of customization in function of the minimum core competences needed, and because it would facilitate accessibility prior to starting an assignment as a DV.

Opportunities for gaining minimum core competences should be made widely available to veterinarians throughout Europe. In this regard, the establishment of distance learning courses (e.g. webinars, e-learning) should be encouraged and supported. The training courses need not lead to a formal qualification; however, the acquisition of competences should be verified by an exam and records of successful completion of the training course should be maintained. To promote the mobility of DVs across Europe, introductory training courses should be designed according to learning outcomes that are defined and regularly reviewed by a European veterinary body composed of experts in the laboratory animal medicine and science field, and are mutually approved by the European Member States.
ESLAV and ECLAM are well recognized professional associations with expertise in this field and could therefore take a role in this framework.

Depending on the size, purpose and complexity of the establishment, and also according to the needs of the veterinarian, further competences could then be gained. This will certainly allow the veterinarian to gain respect from colleagues working at their establishment and to perform their role with authority.

Veterinarians who provide input and advice to the ethical review of project proposals should have sufficient knowledge and expertise in the following areas: (i) competences in laboratory animal medicine regarding the species involved; (ii) knowledge about the models and procedures to be used in projects, and how these may impact on animal well-being; (iii) a sound understanding of the concept of harm/benefit analysis and of the assessment of severity; (iv) expertise on sourcing animals (including breeding and quality aspects such as genetics and microbiological quality and standardization) and their husbandry and care; (v) how any deviations from the standards in Annex III of Directive 2010/63/EU (for scientific reasons) would impact animal welfare and study outcome; (vi) application of the principles of the 3Rs based on contemporary best practices; and (vii) a sound understanding of experimental design and procedures resulting from experience as a researcher. In-depth specific training in these topics would therefore be helpful.

The quality of any training programme depends on the competence of those engaged in the establishment of training standards and in the delivery of the training. The assessment of competences requires additional training that is typically not part of the veterinary undergraduate training programmes. It is recommended that suitable training on how to assess competences be obtained from academia, online training courses, etc. where needed. LAVs should be able to demonstrate personal competences in these training topics.

Proposed training framework for a tiered approach to professional development

A tiered approach to postgraduate veterinary training in laboratory animal medicine and science offers a professional and career development pathway that can be combined with jobs of increasing complexity and responsibilities (Figure 1). For instance, a veterinarian that is relatively new to the field could enrol in a training programme leading to a mid-tier level qualification such as a Master or a Certificate in Laboratory Animal Science. As their career develops, a higher laboratory animal medicine and science qualification may be desired for LAVs working at larger establishments where a large variety of species are kept or where the type of work involved at the establishment is particularly complex, or subject to rapid change, or taking on high-level managerial responsibilities. Options for further development to the specialist level include a National Diploma, or an ECLAM Diploma which constitutes the highest European qualification in this field. Diplomates may be particularly well suited to provide advice for the review of complex projects that require in-depth understanding of the importance of good scientific design and the minimization of the occurrence of bias.

VetCEE is a European accreditation scheme for mid-tier level veterinary postgraduate continuous education programmes addressed to practising veterinarians in different areas of the veterinary profession. It was recently set up as a joint initiative of EAEVE, European Body of Veterinary Specialisation (EBVS, http://www.ebvs.org/), FVE and Union of the European Veterinary Practitioners (UEVP, http://www.fve.org/). Its remit is to promote harmonization of lifelong learning for veterinarians, to ensure quality education at the mid-tier level, and to facilitate mutual recognition of the different training schemes between the different European countries. VetCEE, based on input from experts in the field, could provide the basis for a harmonized approach to veterinary continuing education in laboratory animal medicine and science in Europe between the DV minimum level as defined above and the ECLAM Diplomate level.

Continuing professional development of laboratory animal veterinarians

Veterinarians should take part in continuing professional development (CPD) in order to maintain and develop the level of knowledge and skills relevant to their work. Advanced training opportunities discussed above represent a formal conduit to ensure CPD. However, CPD can also be gained through attendance and/or participation in scientific meetings organized by professional veterinary organizations.

The online survey conducted by ESLAV and ECLAM between April and June 2012 revealed that 90% of responders had access to CPD in their current position and expressed a strong interest in taking part in CPD on various topics to gain in-depth knowledge. However, it also showed that budget constraints were seen as the main hurdle in receiving CPD.

It is important that employers recognize the need for CPD of LAVs as the acquired skills and knowledge will certainly be beneficial to the establishment. Therefore they should allow not only sufficient time but also the means to participate in CPD. When working as a
veterinary continuing education in laboratory animal medicine and science in Europe between the DV minimum level as defined above and the ECLAM Diplomate level.

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Conclusion

DVs together with LAVs play an important role in ensuring high standards for the care and use of laboratory animals. The field of laboratory animal medicine and science is currently not systematically and consistently included in the veterinary curriculum in Europe, and additional postgraduate training is likely to be needed to ensure that adequate competences are acquired. A basic task-specific DV module should be developed on the basis of learning outcomes that are mutually recognized across Europe to ensure that DVs

Figure 1. Routes for a tiered approach to training and education in laboratory animal medicine and science. Note: FELASA categories refer to currently available FELASA-accredited courses.11,13
Safety studies of post-surgical buprenorphine therapy for mice


Abstract
The use of appropriate analgesia in laboratory mice may be suboptimal because of concerns about adverse events (AE). Target Animal Safety trials were conducted to determine the safety of an extended-release suspension of buprenorphine. Drug or control suspensions were injected subcutaneously in surgically-treated BALB/c mice anesthetized with ketamine–xylazine to mimic post-operative conditions in which the compound might commonly be administered. Single and repeat five-fold ($5\times$) excesses of the 3.25 mg/kg intended dose were used to provoke potential AE. Trials included prospective measurements of weight changes, blood chemistry, hematology, and histopathology. Clinical and histopathology findings were similar in drug-treated and control mice in a four-day trial using a single 16.25 mg/kg, $5\times$ overdose of the drug. In a 12-day trial, which used a total buprenorphine dose of 48.75 mg/kg, clinical and histopathology values were also similar in control and drug-treated female mice. In the male arm of the repeat-overdose trial, two of eight mice died on the morning of day 12, three days following the third 16.25 mg/kg overdose administration. Histopathology did not reveal a cause of death. In a 14-month trial using a single 3.25 mg/kg dose of the drug, no significant findings identified potential AE. These findings indicate a high tolerance to an extended-release buprenorphine suspension administered post-operatively in mice with appropriate husbandry.

Keywords
analgesia, mice, buprenorphine, adverse events, extended-release
opiates on weight gain and gastric distress in laboratory rodents. Rates of post-surgical weight gain frequently serve as markers for recovery. While appetite suppression is common in the surgical recovery process, there are reports of morbid gastric stress in opiate-treated rodents secondary to pica. Growing evidence indicates that management issues can be addressed by the use of long-acting analgesic implants, and, perhaps, by using food-based pharmaceuticals. Clark and coworkers have demonstrated that the morbidity effects of pica in buprenorphine-treated rats are exacerbated by wood chip bedding and could be mitigated by appropriate husbandry. We hypothesized that an extended-release suspension of buprenorphine could be used safely in mice housed on soft contact bedding. Buprenorphine has a ceiling effect on respiratory depression and there is considerable evidence that its use in veterinary medicine is safe.

To examine this hypothesis, surgically-treated male and female mice were challenged with significant overdoses of a proprietary buprenorphine suspension and monitored for AE according to US Food and Drug Administration (FDA) guidelines for assessing the safety of veterinary pharmaceutical products. Similar guidelines are used by European regulatory authorities to determine the effects of drugs on major organ systems in target animal species. Hematology and serum chemistry values were measured in mice treated with a surgical procedure using ketamine–xylazine (KX) anesthesia and challenged by single and repeat trials using one to five-fold doses (3.25–48.75 mg/kg) of an injectable buprenorphine suspension. Body weights, organ weights, and histopathology were performed on all mice. All mice were subjected to a surgical procedure (see Materials and methods). KX anesthesia was used based on 20 years of experience of the Johns Hopkins Hunterian Laboratories in implanting long-acting drug delivery systems in mice and rats.

The results of these trials demonstrate that an extended-release lipid–buprenorphine suspension can be used safely in male and female (20–22 g) adult BALB/c mice for analgesia following a surgical procedure. We believe this is the first study of a drug specifically designed for the management of pain in mice.

Materials and methods

Animals

Studies were approved by the Johns Hopkins University (JHU) Institutional Animal Care and Use Committee. The study was carried out in the JHU Department of Molecular and Comparative Pathobiology (Baltimore, MD, USA). Male and female BALB/c (6–8 weeks old and weighing 20–22 g) mice were obtained from Charles River Laboratories (Wilmington, MA, USA). The mice were inspected for general health conditions before being housed at a density of 4–5 mice per cage in Smart Bio-Pak cages (Allentown, NJ, USA) with Tek-Fresh bedding (Harlan, Madison, WI, USA), and were allowed free access to Teklad Global Rodent Diet chow (Harlan) and Baltimore City water. Mice were held for 14 months to enable complete studies using older mice. A total of 36 female and 36 male mice were used for the four-day, 12-day, and 14-month studies described below.

Study design

The study design was based on Target Animal Safety (TAS) protocol guidance to determine the safety of a generic drug. The bioequivalent target range was selected from published reports demonstrating that buprenorphine blood levels greater than 0.5 ng/mL produce positive tail-flick responses in mice, thermal latency in dogs, and responses in human volunteers. In a series of dose finding studies, male and female mice were injected with increasing doses of a lipid–drug suspension containing up to 25 mg/kg buprenorphine. A dose of 3.25 mg/kg, which afforded blood level concentrations of more than 1 ng buprenorphine/mL for at least two days, was selected for the TAS study.

In TAS studies, the mice were housed one per cage. For statistical purposes the experimental unit was the cage. No significant differences in weight, hematology, clinical chemistry, and clinical observations, were observed in the male and female mice dosed at 0 (control), 3.25, and 9.75 mg/kg in pilot studies. Thus, safety studies were conducted with five-fold excesses of the intended dose. The study period was four days, being one day more than the three-day elimination period. Eight male and eight female mice per group were used in the first TAS study comparing a 0× (control) and a 5× dose (16.25 mg/kg) challenge. Eight male and female mice per group were used in the second TAS study comparing 0× and 5× doses repeated at three four-day intervals. In a 14-month study, four male and four female mice were injected with a 1×, 3.25 mg/kg dose of the drug. No vehicle controls were used in this trial. Parameters evaluated in the four- and 12-day trials included body weight, hematology, clinical chemistry, clinical observations, and gross and histopathology. Daily clinical observations were not performed in the 14-month trial. The hypothesis tested was that the data for these
parameters would be different in mice with 0× and 5× doses of such drugs.

**Trial structure**

- **Single 0 and 5× doses, four-day trial:** In the four-day trial, eight mice of each gender were anesthetized, subjected to a sham surgical procedure, and dosed on day 0 with single 0 and 5× doses (16.25 mg/kg) of the drug, or the same volume (0.250 mL) of the control preparation.

- **Repeat, three 0 and 5× doses, 12-day trial:** In the 12-day dose-repeat trial, eight mice of each gender were anesthetized, subjected to a sham surgical procedure, and dosed with 0 and 5× doses (16.25 mg/kg) of the drug, or the control suspension, on days 0, 4, and 8.

- **Single 1× dose, 14-month trial:** In the 14-month trial, four mice of each gender were anesthetized, subjected to a sham surgical procedure, and dosed on day 0 with a single 1× dose (3.25 mg/kg) of the drug. No vehicle control was used in this trial.

The mice in the single-dose and repeat-dose trials were evaluated by daily clinical observations for signs of distress by an observer female veterinarian who was blinded to the treatment groups. At the midpoint of the first two trials, day 2 or day 6, half of the mice were weighed, euthanized, and then exsanguinated to collect blood for hematology and clinical chemistry testing. At the endpoint of each trial, day 4, day 12, and month 14, the remaining mice were euthanized to measure body weight, hematology, clinical chemistry, and anatomical pathology.

- **Coagulation studies:** A 12-day dose-repeat trial with eight control and eight female mice treated with a 48.75 mg/kg dose, the highest dose to be tested, was conducted to furnish sufficient blood for coagulation studies. Mice in this study were not examined by histopathology or daily clinical observations.

**Drug delivery**

Injectable suspensions of the drug powder and the control were prepared by suspending 80 mg of powder per milliliter of medium chain triglyceride (MCT) oil (Miglyol 812; Sasol, Hamburg, Germany) followed by a brief shaking to make a homogeneous suspension containing approximately 4 mg of powder per 0.05 mL of MCT oil. Suspensions were generally prepared within 1–2 days of use and stored at 2–8°C. A single (1×) dose consisted of a 0.05 mL drug suspension. In the study comparing 0 and 5× doses, the control groups were given 0.25 mL of the control suspension. One-milliliter syringes with 1 inch 20 gauge needles were used to inject suspensions (described below) of the cholesterol–triglyceride–buprenorphine powder and the cholesterol–triglyceride control powder.

**Buprenorphine blood concentration measurements**

Blood concentrations of buprenorphine were measured by an LC–MS/MS method (described below). Eight-week-old male and female mice (24 per sex) and housed three per cage were anesthetized and injected with a 3.25 mg/kg dose of the drug. Cages were changed daily to prevent re-dosing by coprophagy. Because the LC–MS/MS assay required 0.25 mL of plasma, mice were euthanized at the time points reported in ‘Results’. Blood samples were collected at 6 h and subsequent 23–25 h intervals after the time of the SC analgesic injection. Day 0 values were obtained from untreated male and female BALB/c mice weighing 20–22 g.

**Surgical procedure**

The surgical procedure used was based on the procedure used to implant Alzet mini-osmotic pumps in mice and rats. A video of the procedure, which is briefly described below, is available at the Alzet website.21 Mice were given intraperitoneal (IP) anesthesia with a solution containing 25 mg/mL ketamine plus 2.5 mg/mL xylazine and 14.25% ethanol in saline. The dose of anesthesia was 0.15 mL/20 g per mouse.

When anesthesia was established, approximately 1 cm² of mid dorsal skin, away from the interscapular area, was shaved, washed with ethanol, and then coated with Betadine. Mice were transferred to a procedural table that was cleaned with 70% ethanol solution and covered with a clean disposable towel. A sterile disposable no. 10 blade was used to make a 4–5 mm incision through the skin only. Bleeding, if any, was controlled...
with sterile gauze and light pressure. Sterile forceps were used to separate the skin and to create an approximately 2 x 4 cm subcutaneous pocket. The skin was then apposed and stapled with 9 mm Autoclips (Kent Scientific, Torrington, CT, USA). After the skin was stapled, mice were injected with a drug or control suspension into the interscapular subcutis. All mice in the study were treated to this ‘sham surgical procedure’.

After the procedure, the mice were moved to a holding cage. This cage contained a 37°C heating pad covered with a clean disposable towel. Each mouse was placed in a clean cage after it regained consciousness, as demonstrated by movement and the absence of signs of distress, which included, but were not limited to, sluggish movement, abnormal paw movements, efforts to scratch the incision site, and cowering in a corner of the holding cage.

**Euthanasia**

The mice were asphyxiated with carbon dioxide. After a one-minute absence of respiration they were weighed. The heart was exposed. The mice were exsanguinated via cardiac puncture to obtain approximately 0.8 mL of blood for hematology and clinical chemistry testing. They were then perfused through the heart with a syringe-driven 10% neutral buffered formalin.

**Clinical observations**

A female veterinarian blinded to the test groups observed the mice between 07:00 and 08:00 h and between 17:00 and 18:00 h daily. Observation forms were designed for the entry of ‘Yes/No’ scores, and numerical grading of signs and symptoms including respiration, tremors, motor activity, movement, nasal and ocular findings, bleeding, and surgical site erythema, edema and infection. Space was available for comments. The same forms were used for both TAS studies: the single-dose four-day observation period, and the three repeated-dose 12-day observation period. Cage conditions, motor activity, ocular findings, and the appearance of fur were observed twice daily.

**Clinical laboratory tests**

Blood chemistry, hematology, and histopathology were performed at the Johns Hopkins Phenotyping Core. Hematology tests were performed on a Hemavet 950 Hematology System (Drew Scientific, Waterbury, CT, USA). Values obtained included white blood cell, neutrophil, lymphocyte, monocyte, eosinophil, basophil, red blood cell and platelet counts; hemoglobin concentration, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, red cell distribution width, and mean platelet volume. A VetACE Clinical Chemistry system (Alfa Wassermann, West Caldwell, NJ, USA) was used to measure blood chemistry profiles: cholesterol (Chol), triglycerides (Tri), uric acid (UA), total bilirubin (TBili), glucose (Gluc), total protein (Tp), calcium (Ca), urea nitrogen (BUN), creatinine (Creat), albumin (Alb), high density lipoproteins (HDL), direct bilirubin (DBili) and the enzymes creatine kinase (CK), lactate dehydrogenase (LDH), alkaline phosphatase (ALP), amylase (Amy), gamma glutamyl transferase (GGT), alanine aminotransferase (ALT), and aspartate aminotransferase (AST).

Coagulation studies were performed at the JHU Department of Laboratory Medicine within 1–2 h of sample acquisition. Groups of eight female mice were injected with three doses of 5 mg/kg drug suspension at days 0, 4, and 8, or control suspension as described in the repeat, three doses, 12-day trial format. Four mice from each group were euthanized at the midpoint, day 6, and at the endpoint, day 12. Exsanguination provided approximately 0.8 mL of blood that was gently mixed with 0.08 mL of citrate anticoagulant solution.

**Body and organ weights**

The mice were weighed in procedure rooms with a calibrated Taconic mouse scale (Taconic, Hudson, NY, USA) before they were assigned to a treatment group and no more than 24 h before they were injected with one or more doses of the drug or control suspensions on day 0. All subsequent weights were measured blind to the dose group. Mice scheduled for euthanasia were sedated with carbon dioxide and weighed on an electronic Ohaus micro balance (Ohaus, Parsippany, NJ, USA). Tissues were weighed on an Ohaus micro balance.

**Histopathology**

Twenty male and 20 female BALB/c mice, 6–8 weeks old upon arrival, were assigned to five treatment groups as defined in the study protocol (Table 1). Histopathology was performed on endpoint mice as described previously. There were 16 mice in the four-day single 16.25 mg/kg dose and control challenges, 16 mice in the 12-day repeat 48.75 mg/kg dose challenges, and the 14-month four female and four male mice in the single 1 x, 3.25 mg/kg dose test. Heart, kidneys, liver, and spleen were collected and weighed. More than 30 tissues were examined for a total of 13 slides per mouse. The tissue list is summarized in Table 2.

Two male mice in the 12-day repeat 48.75 mg/kg dose trial were found dead on day 12 and were not perfused. Their tissues were collected and examined as for the other animals. After trimming, the tissues were
processed, embedded in paraffin, sectioned, mounted on
glass slides and stained with hematoxylin and eosin
(H&E). Tissues listed in Table 2 were evaluated by
light microscopy.

Statistics
Analyses of treatment group blood concentrations were
made using GraphPad Prism Software version 5.04
(La Jolla, CA, USA). Microsoft Excel version 2007
was used to generate average and standard deviation
(SD) data of hematology and clinical chemistry values.

Graham StarTox version 3.1.0 software was gener-
ously made available (Druquest International, Leeds,
AL, USA) to manage histology data.

Results

Blood concentrations of drug
Plasma concentrations of buprenorphine in mice given
the 3.25 mg/kg (1×) dose of the drug averaged 16 ng/
ml 6 h after SC injection (Table 3). Approximately 1 ng
or more of the drug per milliliter of plasma was present
through day 3. The average plasma concentration
was less than 0.5 ng/mL by day 4. There were no
differences between male and female blood
concentrations.

Clinical observations
Approximately 2640 clinical observation entries were
recorded for the mice in the four-day trial, and 7920
entries were recorded for the mice in the 12-day trial.
There were no significant differences in signs of pain or
distress between male and female mice in the drug treat-
ment and control groups in either trial.

Body weight
In the four-day TAS trial drug-treated female and male
mice lost an average of 9% and 2% body weight by day
4, respectively. The weight losses were not significantly
different from weight losses in the surgically-treated con-
trol mice. The effects of three 5× doses of the drug on
male and female mouse weights in the 12-day trial are
shown in Table 4. The total dose of buprenorphine from
the three doses of the drug was 48.75 mg/kg. Through
day 8 of the 12-day trial, body weights of the male and
female mice in the drug versus control groups were the
same. Body weights of the female mice challenged
with three 5× doses of the drug or control suspension
at four-day intervals were similar at day 12.

Table 1. Summary of experimental design: histopathology phase.

<table>
<thead>
<tr>
<th>Test or control article</th>
<th>Total volume administered</th>
<th>Number of doses</th>
<th>Days of test</th>
<th>Number of animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control suspension</td>
<td>0.25 mL</td>
<td>1</td>
<td>4</td>
<td>4♂, 4♀</td>
</tr>
<tr>
<td>Long-acting drug suspen</td>
<td>0.25 mL</td>
<td>1</td>
<td>4</td>
<td>4♂, 4♀</td>
</tr>
<tr>
<td>Control suspension</td>
<td>0.75 mL</td>
<td>3</td>
<td>12</td>
<td>4♂, 4♀</td>
</tr>
<tr>
<td>Long-acting drug suspen</td>
<td>0.75 mL</td>
<td>3</td>
<td>12</td>
<td>4♂, 4♀</td>
</tr>
<tr>
<td>Control suspension</td>
<td>0.05 mL</td>
<td>1</td>
<td>426</td>
<td>4♂, 4♀</td>
</tr>
</tbody>
</table>

♂: male; ♀: female.

Table 2. Tissues evaluated by light microscopy.

Adrenal gland
Bone with bone marrow, femur
Brain (cerebrum, midbrain, cerebellum, medulla/pons)
Dorsal skin surrounding implant(s)
Epididymis (males)
Eyes (with optic nerves)
Gall bladder
Heart
Kidneys
Large intestine, cecum
Large intestine, colon
Liver
Lung
Lymph nodes*
Mammary glands (females)
Ovaries (females)
Pancreas
Parathyroid gland
Skeletal muscle, biceps femoris
Small intestine, duodenum
Small intestine, jejunum
Small intestine, ileum
Spinal cord with spine
Spleen
Stomach
Ventral skin
Testis (males)
Thyroid (with parathyroid)†
Urinary bladder

*Lymph nodes included submandibular superficial cervical
collected with salivary glands from the neck; mesenteric and
pancreaticoduodenal collected with mesentery and pancreas.
†Parathyroid glands were evaluated when present in the plane of
section of the thyroid gland.
Two male mice were found to be moribund on the morning of day 12. The weights of the two dead mice were approximately 2 g less than those of the other drug-treated or control mice.

Organ weight

Heart, kidney, and spleen weights were similar in the drug and control groups of both sexes in the single-dose four-day trial. On average, livers weighed approximately 0.1 g less in the 5× dose groups in males and females. The differences, however, did not reach statistical significance using *t*-tests. The *P* value for a treatment difference in the male group was 0.129; the value for a difference in the female group was 0.130.

Group mean organ weights of the mice in the 12-day dose-repeat trial are shown in Table 5. Liver weights on average were about 0.1 g greater in the 5× female dose group compared with the control group. The difference was not significant. There were no significant differences in the other organ weights in the female test group. The average liver weight in the two surviving males in the 5× dose-repeat group was similar to that in the dose-repeat control group. Spleen weights in the two surviving males of the 5× dose group were on average 25% higher compared with the control spleens. A similar average increase was seen in the female group. Liver weights in the control and dose groups were approximately 12% higher in the mice receiving three doses of the drug or placebo compared with those receiving a single dose.

**Table 3.** Buprenorphine blood concentration in mice treated with a single dose (3.25 mg/kg).

<table>
<thead>
<tr>
<th>Day</th>
<th>0.25</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma concentration (ng/mL, <em>n</em> = 6)*</td>
<td>16.3 ± 8.3</td>
<td>4.1 ± 4.2</td>
<td>1.3 ± 0.9</td>
<td>1.5 ± 0.7</td>
<td>0.4 ± 0.4</td>
</tr>
</tbody>
</table>

*Three male and three female mice; average ± standard deviation.

**Table 4.** Mean body weights in the 12-day dose-repeat Target Animal Safety trial, 48.75 mg/kg total dose.

<table>
<thead>
<tr>
<th>Day</th>
<th>Female mice</th>
<th>Male mice</th>
<th>Female mice</th>
<th>Male mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control Average ± SD</td>
<td>Three 5× doses Average ± SD</td>
<td>Control Average ± SD</td>
<td>Three 5× doses Average ± SD</td>
</tr>
<tr>
<td>0 (n = 8)</td>
<td>23.6 ± 0.4</td>
<td>23.2 ± 1.0</td>
<td>23.1 ± 2.0</td>
<td>23.3 ± 1.0</td>
</tr>
<tr>
<td>4 (n = 8)</td>
<td>23.1 ± 0.6</td>
<td>23.1 ± 0.2</td>
<td>23.0 ± 2.2</td>
<td>21.8 ± 1.2</td>
</tr>
<tr>
<td>6 (n = 4)</td>
<td>23.2 ± 0.4</td>
<td>22.6 ± 0.6</td>
<td>24.3 ± 1.4</td>
<td>22.4 ± 1.2</td>
</tr>
<tr>
<td>8 (n = 4)</td>
<td>23.8 ± 1.0</td>
<td>24.3 ± 0.6</td>
<td>23.6 ± 0.4</td>
<td>22.5 ± 0.4</td>
</tr>
<tr>
<td>12*</td>
<td>23.7 ± 0.6</td>
<td>24.1 ± 0.6</td>
<td>24.4 ± 1.0</td>
<td>23.9 ± 0.8</td>
</tr>
<tr>
<td>12†</td>
<td></td>
<td></td>
<td>21.1 ± 0.3</td>
<td></td>
</tr>
</tbody>
</table>

*Four mice in the female 5× dose and placebo groups; four mice in the male placebo group and two mice in male 5× dose group on day 12.†Weights of two dead mice in the 5× dose group on day 12. SD: standard deviation.

**Table 5.** Mean organ weights in the 12-day dose-repeat Target Animal Safety trial.

<table>
<thead>
<tr>
<th>Organ weight</th>
<th>Female mice</th>
<th>Male mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (<em>n</em> = 4) Average ± SD</td>
<td>Three 5× doses (<em>n</em> = 4) Average ± SD</td>
</tr>
<tr>
<td>Liver</td>
<td>1.40 ± 0.10</td>
<td>1.53 ± 0.07</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.12 ± 0.02</td>
<td>0.14 ± 0.01</td>
</tr>
<tr>
<td>Heart</td>
<td>0.17 ± 0.02</td>
<td>0.19 ± 0.02</td>
</tr>
<tr>
<td>Rt kidney</td>
<td>0.19 ± 0.02</td>
<td>0.20 ± 0.01</td>
</tr>
<tr>
<td>Lt kidney</td>
<td>0.19 ± 0.02</td>
<td>0.19 ± 0.01</td>
</tr>
</tbody>
</table>

SD: standard deviation; Rt: right; Lt: left.
Clinical pathology

There were no differences between hematology, coagulation, and serum chemistry values in the drug and control groups (Tables 6–8).

Histopathology

Day 4, single 5x dose. Minimal Kupffer cell hypertrophy was present in four of four males and in four of four females, but not in the control animals. This finding, which may represent a response to the physical nature of the drug as a suspension rather than as a solution, was considered to be non-adverse. Macrophage infiltrates in lymph node sinuses (sinus histiocytosis) were more prominent in treated than in control animals and were also considered to be a non-adverse response to the physical nature (suspension) of the test article.

Mild lymphoid hyperplasia in mandibular lymph nodes was present in one of four males (mild) and none of the four females in the four-day single-dose control group, and was present (minimal to mild) in three of four males and in three of four females in the four-day single dose test article group. Because this occurred in the region of the incision where the drug was administered it was considered to be a non-adverse response to the sham surgical procedure.

Day 12, repeat 5x dose. No histological findings were attributed to the drug suspension article in the 12-day repeat-dose groups. By day 12, Kupffer cell hypertrophy in the liver, lymphoid hyperplasia in the mandibular and mesenteric lymph nodes and spleen, sinusoidal macrophage infiltrates in the mandibular and mesenteric lymph nodes, and hematopoietic hyperplasia in the bone marrow and spleen had generally similar incidences and severities in the control and test groups. These findings were considered to be due to the administration procedure (dorsal skin incision) of the control and the drug. Minimal or mild findings in other tissues were consistent with spontaneous findings in naïve mice, with no drug-related incidences or severities. These findings were considered to be incidental and unrelated to the drug.

Month 14, single 1x dose. When compared with the controls and the 5x dosed mice from both the four-day and 12-day trials, no histological findings attributed to the drug suspension article were identified in the 14-month single dose group. Mild histiocytic inflammation with cholesterol clefts was present at the surgical site of two single-dosed females and this was likely to have been associated with the surgical technique. Other incidental findings included a focal pulmonary adenoma (1 mm) in the lungs of an individual male and mild small intestinal amyloidosis in two females. These findings are relatively common in aged BALB/c mice.

Table 6. Summary, 12-day 3-dose (48.75 mg/kg) and 14-month 3.25 mg/kg dose challenge: endpoint hematology.

<table>
<thead>
<tr>
<th>Hematology</th>
<th>12-day control, all mice (n=8)</th>
<th>12-day three 5x doses, all mice (n=6)</th>
<th>14-month 1x dose, all mice (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC [K/uL]</td>
<td>Average: 6.0 SD: 2.1</td>
<td>Average*: 6.0 SD*: 4.4</td>
<td>Average: 4.8 SD: 1.6</td>
</tr>
<tr>
<td>NE [K/uL]</td>
<td>1.7 0.5</td>
<td>2.8 3.0</td>
<td>1.3 0.6</td>
</tr>
<tr>
<td>LY [K/uL]</td>
<td>3.4 1.2</td>
<td>2.4 1.2</td>
<td>3.0 1.0</td>
</tr>
<tr>
<td>MO [K/uL]</td>
<td>0.8 0.4</td>
<td>0.7 0.2</td>
<td>0.5 0.1</td>
</tr>
<tr>
<td>EO [K/uL]</td>
<td>0.1 0.1</td>
<td>0.1 0.1</td>
<td>0.1 0.1</td>
</tr>
<tr>
<td>BA [K/uL]</td>
<td>0.0 0.1</td>
<td>0.0 0.0</td>
<td>0 0</td>
</tr>
<tr>
<td>RBC [M/uL]</td>
<td>9.8 0.4</td>
<td>9.3 0.4</td>
<td>10.1 0.4</td>
</tr>
<tr>
<td>Hb [g/dL]</td>
<td>14.5 0.6</td>
<td>13.8 0.9</td>
<td>14.8 0.4</td>
</tr>
<tr>
<td>HCT [%]</td>
<td>53.6 3.0</td>
<td>51.7 2.7</td>
<td>52.8 1.9</td>
</tr>
<tr>
<td>MCV [fL]</td>
<td>54.9 1.5</td>
<td>55.7 0.9</td>
<td>52.2 1.0</td>
</tr>
<tr>
<td>MCH [pg]</td>
<td>14.8 0.4</td>
<td>14.8 0.7</td>
<td>14.7 0.5</td>
</tr>
<tr>
<td>MCHC [g/dL]</td>
<td>27.0 0.7</td>
<td>26.6 1.4</td>
<td>28.1 0.8</td>
</tr>
<tr>
<td>RDW [%]</td>
<td>18.8 0.8</td>
<td>19.7 1.8</td>
<td>21.4 1.0</td>
</tr>
<tr>
<td>PLT [K/uL]</td>
<td>895.0 73.9</td>
<td>893.8 59.8</td>
<td>701 170</td>
</tr>
<tr>
<td>MPV [fL]</td>
<td>5.5 0.1</td>
<td>5.6 0.2</td>
<td>5.4 0.1</td>
</tr>
</tbody>
</table>

*Four female and two male mice. SD: standard deviation; WBC: white blood cell; NE: neutrophil; LY: lymphocyte; MO: monocyte; EO: eosinophil; BA: basophil; RBC: red blood cell; Hb: hemoglobin; HCT: hematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; RDW: red cell distribution width; PLT: platelet; MPV: mean platelet volume.
mice and were considered to be spontaneous and unrelated to the drug.

**Discussion**

The present report describes the morbidity and mortality encountered in a TAS trial of an extended-release buprenorphine veterinary pharmaceutical product for moderate to severe pain therapy in surgically-treated mice. A sensitive and specific LC–MS/MS analysis demonstrated that a single, 3.25 mg/kg dose of the drug injected at the time of surgery afforded plasma concentrations of the drug of 1 ng/mL or more for 2–3 days (Table 3). These concentrations have been

<table>
<thead>
<tr>
<th>Table 7. Clotting factors and buprenorphine levels in female mice treated with three 5× doses of drug for mice.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor</td>
</tr>
<tr>
<td>--------------------------------------</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Fibrinogen (mg/dL)</td>
</tr>
<tr>
<td>PT (s)</td>
</tr>
<tr>
<td>aPTT (s)</td>
</tr>
</tbody>
</table>

Table 8. Twelve-day 3-dose and 14-month 1× challenge: endpoint serum chemistry.

<table>
<thead>
<tr>
<th>Chemistry</th>
<th>Control, all mice (n=8)</th>
<th>5× dose, all mice (n=6)*</th>
<th>14-month 1× dose, all mice (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average SD</td>
<td>Average SD</td>
<td>Average SD</td>
</tr>
<tr>
<td>Chol (mg/dL)</td>
<td>147.8 23.0</td>
<td>129.7 35.7</td>
<td>170.0 44.4</td>
</tr>
<tr>
<td>Tri (mg/dL)</td>
<td>145.5 35.9</td>
<td>127.2 25.8</td>
<td>142.1 50.4</td>
</tr>
<tr>
<td>UA (mg/dL)</td>
<td>2.8 0.7</td>
<td>2.9 0.7</td>
<td>2.4 0.6</td>
</tr>
<tr>
<td>CK (U/L)</td>
<td>261.5 231.6</td>
<td>197.2 117.1</td>
<td>118.9 65.3</td>
</tr>
<tr>
<td>GGT (U/L)</td>
<td>4.9 0.6</td>
<td>5.5 0.8</td>
<td>6.5 1.9</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>44.1 11.3</td>
<td>34.3 4.3</td>
<td>52 10.2</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>77.6 36.2</td>
<td>71.3 18.0</td>
<td>72.9 9.6</td>
</tr>
<tr>
<td>Amy (U/L)</td>
<td>866.3 60.3</td>
<td>897.0 102.8</td>
<td>1029.3 118.0</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>121.6 30.0</td>
<td>103.8 34.9</td>
<td>79.8 5.6</td>
</tr>
<tr>
<td>TBili (mg/dL)</td>
<td>0.2 0.0</td>
<td>0.3 0.1</td>
<td>0.3 0.1</td>
</tr>
<tr>
<td>Glu (mg/dL)</td>
<td>243.5 52.4</td>
<td>202.2 15.4</td>
<td>207.9 27.9</td>
</tr>
<tr>
<td>Tpr (mg/dL)</td>
<td>4.9 0.3</td>
<td>4.8 0.2</td>
<td>5.8 0.2</td>
</tr>
<tr>
<td>Ca (mg/dL)</td>
<td>9.9 0.6</td>
<td>9.8 0.2</td>
<td>9.9 0.2</td>
</tr>
<tr>
<td>BUN (mg/dL)</td>
<td>14.0 1.2</td>
<td>13.8 3.4</td>
<td>13.9 2.2</td>
</tr>
<tr>
<td>Creat (mg/dL)</td>
<td>0.4 0.2</td>
<td>0.3 0.1</td>
<td>0.4 0.1</td>
</tr>
<tr>
<td>Alb (mg/dL)</td>
<td>3.1 0.1</td>
<td>3.0 0.1</td>
<td>3.2 0.1</td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>99.0 21.9</td>
<td>84.0 29.9</td>
<td>82.9 26.5</td>
</tr>
<tr>
<td>LDH (U/L)</td>
<td>211.1 42.4</td>
<td>147.3 37.6</td>
<td>188.1 15.1</td>
</tr>
<tr>
<td>DBili (mg/dL)</td>
<td>0.2 0.0</td>
<td>0.2 0.2</td>
<td>0.3 0.1</td>
</tr>
</tbody>
</table>

*Four female and two male mice. SD: standard deviation; Chol: cholesterol; Tri: triglycerides; UA: uric acid; CK: creatine kinase; GGT: gamma glutamyl transferase; ALT: alanine aminotransferase; AST: aspartate aminotransferase; Amy: amylase; ALP: alkaline phosphatase; TBili: total bilirubin; Glu: glucose; Tpr: total protein; Ca: calcium; BUN: urea nitrogen; Creat: creatinine; Alb: albumin; HDL: high density lipoproteins; LDH: lactic dehydrogenase; DBili: direct bilirubin.
consistently associated with pain therapy in animal and human studies. In separate studies, tests to measure efficacy using Hargreaves plantar analgesia methods showed that drug-treated female mice averaged about 15% more latency than did controls for four days. Significant latency responses were observed using tail-flick studies. Drug-treated male and female mice showed 2–3 fold slower tail-flick responses for two days.

Post-operative buprenorphine therapy has been associated with weight loss. However, it appears possible that stress caused by repeated SC and IP injections could also play a role in suppressing appetite and weight gain. Gastric distress independent of pica involving hardwood bedding may also be involved. We saw no significant weight changes in control mice or mice dosed with 5× doses of the drug for at least eight days (Table 4). There were no significant weight changes in the heart, liver, spleen or kidney in male and female mice receiving 16.25 mg/kg of buprenorphine, and in female mice dosed with 48.75 mg/kg of the drug (Table 5). The necropsy studies showed no evidence of ingested bedding. The present results support the theory that stresses associated with repeated buprenorphine injections may be partially responsible for the reported weight losses. Nonetheless, the trend to weight loss in the four-day TAS trial indicates that buprenorphine itself can briefly suppress appetites in surgically-treated mice. A long-acting opiate preparation cannot eliminate concerns about weight gain. Further research may show that different surgical models and other strains of mice are more susceptible to appetite suppression. However, there is little evidence that opiate-associated weight loss in post-surgical therapy is clinically significant or challenging to research models with appropriate husbandry.

Prolonged administration of buprenorphine can decrease white blood cell counts in mice. Mice given daily IP injections of buprenorphine (300 μg/kg) for 60 consecutive days developed severe leukopenia. Leukocytes (WBC) returned to normal ranges within 45 days following withdrawal of the drug. The present study, no AE were observed in hematology studies. Blood counts, differentials, and hemoglobin measurements from the drug overdose and the carrier solution were similar in the two trials. Platelet counts and volumes were normal in mice treated three times with 5× doses of the drug (Table 6).

Additional information about the effects of the drug on hemostasis was obtained from female mice treated with repeat 5× doses of the drug in a 12-day trial. Fibrinogen levels, prothrombin time (PT), and activated partial thromboplastin time (aPTT) were analyzed at the midpoint of the trial and at day 12. Clotting factors were similar for the treated and control animals at both time points (Table 7). Although fibrinogen concentrations were abnormally low at day 12 in the control and 5× drug groups, repeated 5× challenge doses of the drug did not appear to affect coagulation parameters.

Because buprenorphine is metabolized in the liver, ALT, AST, and other liver enzyme concentrations can offer sensitive markers of buprenorphine hepatotoxicity. Average ALT and AST elevations were found in the 5× dose group of male and female mice at day 2 following the surgical procedure. Yet in the present trials the increases were within the normal range and were comparable with control data. At day 4 in the single 5× overdose trial, chemistry values were similar in the control and drug-treated groups. There was no evidence to support previous reports associating buprenorphine with adverse metabolic effects. Liver chemistry values remained within normal limits in the second TAS trial (Table 8). Although two male mice in the dose-repeat group died at day 12, laboratory chemistries were normal at day 6, or two days after the mice received two 5× doses, or a total of 32.5 mg/kg of the drug in four days. Laboratory values for the two remaining male mice in the 12-day trial were within normal limits (Table 8).

Under the conditions of this study, there were no drug-related gross pathology findings. There was no evidence of skin lesions in any of the study groups. No histological findings in the tissues evaluated, including injection site skin, were attributed to the drug at 16.25 mg/kg. Average organ weight differences between the drug-treated and control mice in the four-day trial were not significant, and were not seen in the 12-day trial, which further indicated that differences observed in the four-day trial were as likely due to chance as a treatment effect. Histological findings attributed to the drug in the single-dose groups (day 4) included minimal hypertrophy of hepatic Kupffer cells, and minimal to mild inflammatory changes in all groups. Histopathology revealed that the combined volume MCT oil carrier solutions in the endpoint, day 12 mice, which totaled 0.75 mL, resulted in an MCT oil pool in dorsal SC spaces. The residual oil may account for the mild granulomatous inflammation noted in this site.

Generally mild granulomatous or pyogranulomatous inflammation, sometimes with acicular clefs and lipid-like materials attributed to injected material, was identified in all groups. Histology findings in the injection site at the skin, and increased hematopoiesis in bone marrow and spleen, had similar incidences in the control and drug groups and were considered to be responses to the dosing procedure and to be unrelated to the drug suspension. Findings in other tissues were consistent with spontaneous findings in naïve
mice, and incidences or severities were not drug-related. These findings were considered to be incidental and unrelated to the drug suspension. Epicardial mineralization was present in one of four females exposed to the drug in both the four-day and 14-month single-dose trials, and in the repeated-dose groups. Based on this incidence and because it correlated with a common spontaneous finding in BALB/c mice, mineralization in the heart was considered to be incidental and unrelated to the drug suspension.

Histopathology of the two male mice that died on day 12 did not identify a cause of death. Autolysis compromised evaluation of several tissues. Histopathology findings were comparable with the non-drug treated control mice. Mortality in the 48.75 mg/kg dose group may have been related to hypotension caused by the combination of repeated KX anesthesia plus the high doses of buprenorphine. Roughan and colleagues have described a high mortality rate in rats that received weekly doses of ketamine/medetomidine anesthesia and 0.5 mg/kg SC of buprenorphine for six weeks.27 KX combinations are widely used for anesthesia in mice and other species. The combination was selected for these trials to recapitulate surgical situations in which buprenorphine would be used for post-operative analgesia. KX combinations can induce bradycardia, cardiac depression, and hypotension. Buitrago and colleagues have tested four combinations of drugs—KX, ketamine–xylazine–acepromazine (KXA), ketamine–xylazine–buprenorphine, and ketamine–xylazine–carprofen for their ability to produce anesthesia in BALB/c mice. Induction time, anesthetic duration, blood pressure, pulse rate, and time to recovery were recorded. The KXA combination produced stable physiological parameters and was associated with the longest duration of anesthesia (40 ± 8 min); immobility was produced in all other groups (38 ± 5 min), but a surgical plane of anesthesia could not be confirmed. All KX anesthetic protocols produced significant hypotension.28

The effects of single and multiple doses of the drug on major organ systems as determined by clinical and histopathology studies are summarized in Table 9.

The safety profile of the extended-release buprenorphine suspension described in this report confirms previous studies of long-acting preparations of morphine in mice,7 lipsome-encapsulated oxymorphone in mice,6 and lipsome-encapsulated oxymorphone in rats.29,30 The data are also consistent with previous studies demonstrating that buprenorphine can be applied safely in commonly used research models.2,31–34

### Funding and Conflicts of interest

J Romero is supported by a fellowship from Genentech organized by the American College of Veterinary Pathologists and Society of Toxicologic Pathology Coalition for Veterinary Pathology Fellows. Ward R Richter generously supplied advice and software for the evaluation of histopathology slides. C Chapleo of Reckitt Benckiser supplied buprenorphine for the early trials. Angela Wu and Gina Wilkerson helped prepare the manuscript. Funding for the present study was supplied by the Maryland Industrial Partnership, a State of Maryland fund to promote the development of products and processes through industry/university research partnerships. K Traul has served as a paid consultant to Animalgesic Labs Inc. M Guarnieri received additional funding from Animalgesic Labs Inc, and holds a significant financial interest in Animalgesic Labs.

### References


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**Table 9.** Summary findings present with drug challenge versus control mice.

<table>
<thead>
<tr>
<th>Clinical data</th>
<th>1x dose 3.25 mg/kg</th>
<th>5x dose 16.25 mg/kg</th>
<th>3 times 5x dose 48.75 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 426</td>
<td>Male &amp; female</td>
<td>Male &amp; female</td>
<td>Male &amp; female</td>
</tr>
<tr>
<td>Weight loss</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Hematology</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Coagulation</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Serum chemistry</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Histopathology</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Mortality</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

| Day 2         | Male & female     | Male & female       | Male & female               |
| Weight loss   | No                | No                  | No                          |
| Hematology    | No                | No                  | No                          |
| Coagulation   | No                | No                  | No                          |
| Serum chemistry | No               | No                  | No                          |
| Histopathology | No               | No                  | No                          |
| Mortality     | No                | No                  | No                          |

| Day 4         | Male & female     | Male & female       | Male & female               |
| Weight loss   | No                | No                  | No                          |
| Hematology    | No                | No                  | No                          |
| Coagulation   | No                | No                  | No                          |
| Serum chemistry | No               | No                  | No                          |
| Histopathology | No               | No                  | No                          |
| Mortality     | No                | No                  | No                          |

| Day 6         | Male & female     | Male & female       | Male & female               |
| Weight loss   | No                | No                  | Yes                         |
| Hematology    | No                | No                  | No                          |
| Coagulation   | No                | No                  | No                          |
| Serum chemistry | No               | No                  | No                          |
| Histopathology | No               | No                  | No                          |
| Mortality     | No                | No                  | No                          |

| Day 12        | Male & female     | Male & female       | Male & female               |
| Weight loss   | No                | No                  | No                          |
| Hematology    | No                | No                  | No                          |
| Coagulation   | No                | No                  | No                          |
| Serum chemistry | No               | No                  | No                          |
| Histopathology | No               | No                  | No                          |
| Mortality     | No                | No                  | No                          |


The effect of midazolam on the recovery quality, recovery time and the minimum alveolar concentration for extubation in the isoflurane-anesthetized pig

S A Kleine¹, J E Quandt¹, E H Hofmeister¹ and J Peroni²

Abstract
There are no reported studies evaluating the effect of midazolam on recovery quality, recovery time or minimum alveolar concentration (MAC) at which extubation occurs (MACextubation). Our hypotheses were that midazolam administered prior to recovery would decrease MACextubation, prolong recovery time but provide a smoother recovery. Sixteen Yorkshire pigs were anesthetized with isoflurane for approximately 5 h. The end-tidal isoflurane concentration was then stabilized at 1.4% for 20 min. Pigs were randomly assigned to receive midazolam or saline. The vaporizer was decreased by 10% every 10 min until extubation. Pigs were declared awake by a blinded observer and were assigned a recovery score by the same observer. Mean MACextubation was not significantly different for pigs receiving saline prior to recovery compared with those pigs receiving midazolam. The overall mean MACextubation for both groups was 0.6 ± 0.4 vol%. Time to extubation was not significantly longer with midazolam (124 ± 36 min) compared with the saline group (96 ± 61 min; P = 0.09). Recovery score was not significantly different between groups (midazolam, 0.86 ± 1.1; saline 0.5 ± 0.5; P = 0.26). In conclusion, midazolam did not affect MACextubation. There was no advantage of administering midazolam in the recovery period when performing step-down titration of isoflurane anesthesia.

Keywords
pharmacology, physiology, anaesthesia, techniques

Pigs often require general anesthesia both for medical care and during research projects. Volatile inhalant anesthetics, such as isoflurane, are commonly used to provide anesthesia. These anesthetics prevent movement and cause unconsciousness. Lack of movement is used to determine the minimum alveolar concentration (MAC) and lack of consciousness is used to determine the MACawake. MAC is the end-tidal concentration required to prevent purposeful movement in response to a noxious stimulus in 50% of patients.¹ MACawake, in humans, is the concentration at which an appropriate response to a command occurs in 50% of patients,¹ and generally represents the concentration at which memory is lost or unconsciousness occurs.²³ In animals, a value for MACawake is difficult to determine due to the impossibility of obtaining an appropriate response to a verbal command. Because of this, MACextubation, which is the concentration at which tracheal extubation occurs,⁴ may be a useful value for anesthetists and may be a reasonable substitute for MACawake in veterinary patients.

While inhalant anesthetics provide immobility and unconsciousness, these drugs also come with significant cardiovascular effects.⁵⁶ Isoflurane causes a
dose-dependent decrease in cardiac output, arterial blood pressure, and stroke volume.5,6 In humans, the MACawake for isoflurane is approximately one-third of the MAC.5,7 This allows the inhalant concentration to be titrated to a lower dose while still preventing awareness. Because the adverse cardiovascular effects are dose-dependent, the decreased inhalant concentration should provide better cardiovascular stability.5,6 In humans, MACextubation to MACratio is greater than MACawake to MACratio, so it is likely that most animals are still unconscious at MACextubation.

The addition of muscle relaxants and analgesics can allow for a decreased inhalant concentration by providing balanced anesthesia (muscle relaxation, analgesia and unconsciousness). Benzodiazepines, such as midazolam, provide muscle relaxation9 and sedation, at a dose of 0.1–0.2 mg/kg.10–12 The effects of midazolam on the cardiovascular system are minimal.9 Studies of the cardiovascular effects of midazolam in swine have shown that this drug causes a mild decrease in heart rate but cardiac output and mean arterial blood pressure remain unchanged.11

Because midazolam has minimal cardiovascular effects and provides muscle relaxation, this drug may be useful as an adjunct to inhalant anesthesia. MAC-sparing effects of midazolam have been reported in various species.13,14 Benzodiazepines, like midazolam, may induce sedation and muscle relaxation by acting on either central or spinal gamma aminobutyric acid type A (GABA_A) receptors.13 Due to these effects, benzodiazepines may reduce MAC and aid in providing balanced anesthesia. Midazolam has been shown to decrease the MAC necessary to abolish all movement (MAC_NM) in isoflurane-anesthetized dogs.13 Addition of midazolam may decrease the isoflurane concentration at which consciousness occurs.14 However, no studies evaluating the effects of midazolam on MAC, MACextubation, or MACawake have been performed in pigs. If midazolam decreases MACawake and MACextubation, the anesthetist may be able to use an even lower concentration of inhalant to prevent consciousness and rejection of the endotracheal tube by administering midazolam to a patient or research subject and to provide a more satisfactory recovery quality. The effect of midazolam on recovery time and quality administered just prior to recovery is unknown in pigs.

The purpose of the study reported here is to determine the MACextubation in pigs. In addition, the effects of the administration of midazolam on MACextubation, recovery time, and recovery quality were evaluated. The hypotheses were that administration of midazolam prior to recovery would lower the MACextubation and provide a more satisfactory recovery but prolong the time to extubation.

Materials and methods

Animals

Sixteen healthy, castrated male Yorkshire pigs (Valley Brook Farm, Madison, GA, USA) were used in the study. They were approximately eight weeks old and weighed between 23 and 31 kg. There was an acclimatization period of seven days prior to the start of the study period. The animals were housed at the Veterinary Bioresources Facility at the University of Georgia, Athens, GA, USA. They were housed in groups of three in pens measuring 4 m x 8.4 m. A 12:12 h light/dark schedule was used. The room temperature was 22.2 ± 2.2°C.

Study design

The study was approved by the Institutional Animal Care and Use Committee. Pigs were premedicated with xylazine (Anased; Lloyd Laboratories, Shenandoah, IA, USA) 1.1 mg/kg, and tiletamine/zolazepam (Telazol; Zoetics, Florham Park, NJ, USA) 2.0–3.0 mg/kg intramuscularly in the epaxial muscles. The pigs then received 1.62–9.26 mg/kg of ketamine intramuscularly (Ketaset; Fort Dodge Animal Health, Madison, NJ, USA) to allow for catheter placement. A 22 gauge, one-inch catheter was placed in the marginal ear vein. Ketamine 0.00–17.05 mg/kg was administered intravenously to effect until orotracheal intubation was achieved. After intubation, buprenorphine (Buprenex; Reckitt Benckiser Healthcare Ltd, Hull, UK) 0.01 mg/kg was administered intramuscularly. The endotracheal tube was then connected to a rebreathing circuit and maintained with isoflurane (Isoflo; Abbott Animal Health, Abbott Park, IL, USA) in 100% oxygen delivered at 1 L/min. Agent-specific, temperature-compensated, calibrated vaporizers were used for the delivery of isoflurane. Intermittent positive pressure ventilation (Multiflow 2002; Hallowell EMC, Pittsfield, MA, USA) was used to maintain a target end-tidal carbon dioxide concentration between 35 and 45 mmHg. End-tidal agent concentrations were sampled via a side stream sampling L-shaped connector attached to both the endotracheal tube and the Y-piece of the anesthesia circuit. End-tidal gas concentrations were continuously measured with a calibrated infrared gas analyzer (POET IQ 602; Criticare Systems, Inc, Waukesha, WI, USA). The gas analyzer was calibrated according to the manufacturer’s recommendations, once a day during the study period. Lactated Ringer’s solution (Hospira, Lake Forest, IL, USA) was administered intravenously at a rate of 10 mL/kg/h. Body temperature (Surgivet Advisor; Smiths Medical, Norwell, MA, USA) was measured continuously by a probe placed in the thoracic portion of the esophagus; and body temperature was maintained between 36.9°C and 38.3°C with a
forced-air warming unit (Bair Hugger Model 505; Arizant, Eden Prairie, MN, USA). Blood pressure was monitored via an indirect oscillometric device (Critikon Dinamap 8300; GE Medical Systems Information Technologies, Tampa, FL, USA) due to difficulty in achieving arterial access for direct blood pressure monitoring. The blood pressure occluding cuff was placed on the antebrachium of each pig. The width of the blood pressure-occluding cuff was 40% of the circumference of the forelimb of each pig. When MAP decreased to <60 mmHg, a single fluid bolus (5 mL of lactated Ringer’s solution per kilogram) was administered. If hypotension did not resolve, dobutamine (DOBUTamine; Hospira) at 5 mcg/kg/min was administered intravenously.

**Determination of MAC_{extubation}**

As part of another study, pigs were anesthetized for the creation and treatment of an aneurysm via femoral artery catheterization and ventral midline celiotomy, respectively. Twenty minutes prior to completion of this procedure, the vaporizer was adjusted to achieve an end-tidal isoflurane concentration of 1.4%. Pigs were randomly assigned by lottery to receive midazolam (Midazolam; West-Ward Pharmaceutical, Eatontown, NJ, USA) 0.2 mg/kg intravenously, or an equivalent volume of saline (1 mL/25 kg intravenously). The target end-tidal concentration was maintained at 1.4% for 20 min, then the test drug was administered to each pig. The isoflurane vaporizer dial setting was decreased by 10%, and each pig was allowed to stabilize at that vaporizer setting for 10 min. During each vaporizer step-down, the end-tidal isoflurane concentration remained stable for at least 10 min. Blood pressure, heart rate, respiratory rate, and end-tidal carbon dioxide concentration were recorded, and then the vaporizer setting was decreased by another 10%. This continued until the pig rejected the endotracheal tube, as evidenced by chewing, swallowing and head movement, and was declared awake by one observer blinded to the drug administered. The \( \text{MAC}_{\text{extubation}} \) was then calculated as mean of the end-tidal concentration before a positive response was obtained and the end-tidal concentration when a positive response was obtained. The recovery was then assigned a quantitative score using a modified version of a previously published scoring system (Appendix). Upon recovery from anesthesia, buprenorphine 0.01 mg/kg was administered intramuscularly.

**Postoperative care**

The animals were carefully monitored during anesthesia recovery. They were monitored hourly for 6 h following recovery from anesthesia. The incision and overall health of the animal was evaluated three times daily for seven days following anesthesia and surgery. At the same time the animals were evaluated for pain and buprenorphine 0.01 mg/kg was administered intramuscularly if the pig displayed any signs of pain.

**Statistics**

Normality was determined using the D’Agostino–Pearson test. Normally-distributed data were compared using an unpaired 2-way \( t \)-test, and non-normally distributed data were compared using a Mann–Whitney \( U \)-test. Number of pigs in each group with a recovery score of 0 was compared using a chi-square test. Significance was set at \( \alpha < 0.05 \).

**Results**

There were no significant differences between groups with regard to weight (midazolam, 27.2 ± 3.3 kg; saline, 27.2 ± 3.7 kg, \( P = 0.93 \)) or duration of anesthesia (midazolam, 314.8 ± 41 min; saline 303.3 ± 33 min, \( P = 0.29 \)). No significant differences were found between groups with regard to dose of intramuscular ketamine (midazolam, 4.2 ± 2.16 mg/kg; saline, 3.24 ± 2.59 mg/kg, \( P = 0.27 \)), dose of intravenous ketamine (midazolam, 6.59 ± 4.95 mg/kg; saline, 3.6 ± 2.51 mg/kg, \( P = 0.15 \)), or total dose of ketamine administered (midazolam, 10.79 ± 4.78 mg/kg; saline, 6.84 ± 4.59 mg/kg, \( P = 0.08 \)). Time to extubation was not significantly longer with midazolam (124 ± 36 min) compared with results from the saline group (96 ± 61 min; \( P = 0.09 \)). Recovery score was not significantly different between groups (midazolam, 0.86 ± 1.1; saline, 0.5 ± 0.5; \( P = 0.26 \)).

Mean \( \text{MAC}_{\text{extubation}} \) was not significantly different for isoflurane-anesthetized pigs receiving saline prior to recovery compared with those pigs receiving midazolam. The \( \text{MAC}_{\text{extubation}} \) for the saline group was 0.65 ± 0.19% and the mean \( \text{MAC}_{\text{extubation}} \) for the midazolam group was 0.55 ± 0.18% (\( P = 0.29 \)). The overall mean \( \text{MAC}_{\text{extubation}} \) for both groups was 0.6 ± 0.4%.

**Discussion**

The results of this study indicate that \( \text{MAC}_{\text{extubation}} \) in isoflurane-anesthetized pigs is 0.6%. Previous studies of isoflurane in pigs have shown that MAC for this inhalant is 1.9–2.04%. Using these previously established MAC values, our study demonstrates that the \( \text{MAC}_{\text{extubation}} \) to MAC ratio in pigs is 0.29–0.34 for isoflurane. This ratio is similar to \( \text{MAC}_{\text{awake}} \) to MAC ratios in humans, where ratios of 0.2–0.35 have been reported, however this is lower than \( \text{MAC}_{\text{extubation}} \)
to MAC ratios of 0.8–1.4. All these ratios differ from dogs, which have a MACawake to MAC ratio for isoflurane of 0.8. Based on these findings, MACawake to MAC and MACextubation to MAC ratios vary among species. The difference in MACextubation to MAC ratios may be attributed to differences in airway sensitivity to endotracheal tubes.

In this study, midazolam, at the reported dose, failed to significantly decrease MACextubation values versus MACextubation for those treated with saline. This indicates that midazolam does not decrease the isoflurane concentration required to prevent extubation. In dogs, administration of midazolam has been shown to have MAC-sparing effects and decrease the isoflurane concentration required to prevent movement. In addition, diazepam, a benzodiazepine, and fentanyl administered simultaneously to isoflurane-anesthetized dogs have been shown to have a greater MAC-sparing effect than fentanyl alone. There have been no studies evaluating the effects of the benzodiazepines (e.g. midazolam) on MAC in pigs.

Evidence regarding the duration of midazolam sedation in pigs is limited. Given that a slow alveolar washout was used to determine MACextubation, it is possible that the potential MAC-sparing effects of midazolam had waned at the time of awakening. The duration of effect of midazolam is approximately 50 min in humans and up to one hour in rats. However, the duration of sedation in alpacas was longer at about 90 min. Despite the potentially short duration of action, studies have shown that midazolam, when used as a premedication agent, reduces MAC by 23% in dogs undergoing ovariohysterectomy. A study evaluating the effects of varying doses of ketamine is shorter than the duration of anesthesia in the current study and there were no differences between the groups with respect to their ketamine dose. To the authors' knowledge the pharmacokinetics of xylazine in pigs has not been determined; however a study in pigs has determined that sedation with tiletamine–zolazepam, ketamine, and xylazine has a duration of 61 min.

The results of this study indicate that midazolam, at the reported dose, had no effect on recovery quality. The quality of recovery is important in order to prevent injury secondary to agitation during awakening. In our study on pigs, it is possible that there was inadequate sedation provided by the midazolam or that the duration of midazolam was too short to extend in the recovery period. However, none of the pigs in this study had adverse events during the recovery period. It is possible that the presence of other drugs, tiletamine–zolazepam, ketamine, or buprenorphine, caused a quiet, calm recovery state in pigs of both groups.

A study evaluating the effects of varying doses of ketamine (up to 20 mg/kg) in combination with xylazine and midazolam resulted in complete recovery, standing with no ataxia, in 92 min, which is much shorter than the duration of anesthesia in the above reported study. Additionally, pigs given xylazine–ketamine–tiletamine/zolazepam for anesthesia had a duration of anesthesia of 61 min which was also significantly shorter than the duration of anesthesia in the current study. Buprenorphine at a dose of 0.01 mg/kg provides analgesia for up to 6 h in pigs. It is possible that this drug played a role in providing smooth recoveries in these pigs, however elimination of analgesic medications in the anesthesia protocol was deemed unethical. To the authors' knowledge, there are no studies evaluating the effect of buprenorphine on recovery time or quality in pigs, so the effects of this drug on the recovery quality are unclear. Additionally, this was a small sample size which may have made it difficult to detect small differences in the recovery score between groups.

A potential limitation to this study is that it is difficult to assess awareness and consciousness in animals. Other studies evaluating MACawake in animals have been performed. Due to differences in airway sensitivity among species and lack of response by pigs to verbal commands, MACextubation was evaluated. For this study, a slow alveolar washout was used. This allowed adequate time for the brain and alveolar
concentrations to equilibrate, and minimized any potential error in determining MACawake\textsuperscript{20} or MACextubation that could result from a fast alveolar washout. Despite our inability to truly assess consciousness in animals, it is useful to know the anesthetic concentration at which a specific species rejects the endotracheal tube. Another limitation of this study is that we were unable to determine MAC for isoflurane in this group of pigs. The pigs used in this study were part of another study and were undergoing surgery for aortic aneurysm formation, which prevented MAC determination during the procedure. However, evaluation of MAC and its relationship to MAC multiples in desflurane-anesthetized dogs have found that the determination of individual MAC for animals used in studies of MAC multiples is unnecessary.\textsuperscript{33}

In conclusion, MACextubation in pigs is 0.6 ± 0.4% for both saline- and midazolam-treated pigs. This study showed that midazolam at the reported dose did not affect MACextubation. There was no advantage of administering midazolam in the recovery period when performing step-down titration of isoflurane anesthesia.

Acknowledgements
The authors would like to thank Merilee Thoresen, Tristan Lewis, and Lori Dressel for technical assistance.

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References
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### Appendix. Scoring system for induction, recovery, and ataxia

<table>
<thead>
<tr>
<th>Score</th>
<th>Induction/Recovery</th>
<th>Ataxia</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Excellent</td>
<td>None</td>
</tr>
<tr>
<td>1</td>
<td>Good</td>
<td>Minimal</td>
</tr>
<tr>
<td>2</td>
<td>Fair</td>
<td>Moderate</td>
</tr>
<tr>
<td>3</td>
<td>Poor</td>
<td>Marked</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smooth, calm, uncomplicated</td>
<td>None</td>
</tr>
<tr>
<td>Minimal vocalization and/or struggling</td>
<td>Minimal</td>
</tr>
<tr>
<td>Moderate vocalization and/or struggling</td>
<td>Moderate</td>
</tr>
<tr>
<td>Marked vocalization and/or struggling</td>
<td>Marked</td>
</tr>
</tbody>
</table>
Using the mouse grimace scale to assess pain associated with routine ear notching and the effect of analgesia in laboratory mice

AL Miller and MC Leach

Abstract
Social housing is recommended where possible for laboratory mice. In order to achieve this, mice must be individually identifiable. Although, various methods are available, permanent identification is often required, such as ear notching. This method is likely to be painful and to date there is limited literature on pain assessment and alleviation for this routine husbandry practice. Here we aimed to determine if the mouse grimace scale (MGS) could be used to assess pain in C57BL/6 mice following routine ear notching. Langford et al. found that very acute noxious stimuli (i.e. < 10 min in duration) did not produce a change in MGS score in comparison to baseline. Here, no significant difference was found between MGS scores at baseline and immediately post ear notching, potentially indicating that the pain associated with ear notching is either too acute to assess using the MGS tool or the practice is not painful. Studies in other species indicate that ear notching is painful, therefore, unless we can confidently conclude that the process of ear notching is not painful, we should err on the side of caution and assume it is painful due to the large number of mice ear-notched and potential welfare consequences. Alternative methods of assessing pain following this routine practice should be used in order to assess both the potential pain in mice, and the effectiveness of analgesics or local anaesthetics to relieve any associated pain.

Keywords
mouse, pain, ear notch, mouse grimace scale

Over three million mice were used in scientific procedures in the UK in 2012 with 64% of these being genetically-modified (GM) or harmful mutants. As a social species, it is recommended that mice are group-housed wherever possible to promote welfare, thus some method of identification of individuals within a group is essential to allow for independent tracking throughout studies. Permanent methods of identification, such as ear notching, ear tattooing, ear tagging or implantation of a subcutaneous transponder, are often used rather than semi-permanent methods such as tail marking or hair clipping, especially in long-term studies to limit errors.

Pain and stress resulting from ear notching has been studied in some species. Ear notching in piglets induces pain-related behaviours (e.g. head shaking, shivering and grunting), non-specific behaviours and an increase circulating lactate levels. Ear notching has also been shown to increase heart rate and blood pressure in rats, predominantly in the first hour post procedure.

Ear notching is a technique that has been in regular use for over 75 years in laboratories. It is considered a routine husbandry practice for identification of mice in the laboratory but is also commonly used to collect samples in order to genotype mice. Ear notching is likely to be painful in mice, however to date, very limited data have been published on pain assessment and alleviation in this species. Due to the large numbers

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of mice requiring permanent identification and/or to be genotyped, this is an important issue to address as pain not only compromises welfare, but also potentially the validity of the data collected from these animals.

The mouse grimace scale (MGS) developed by Langford et al.\textsuperscript{12} is a mouse pain assessment tool found to be useful in a range of laboratory procedures.\textsuperscript{13,14} It has been suggested to be most effective at detecting pain that is present for more than 10 min, but less than 4 h.\textsuperscript{12} The process of ear notching involves application of very acute noxious stimulus, however pain associated with the process may be present for a period following the procedure as indicated by changes in heart rate and blood pressure in the rat.\textsuperscript{7}

Eutectic mixture of local anaesthetics (EMLA) cream can easily be applied to provide topical anaesthesia. EMLA cream has been used in a range of species to reduce or prevent pain associated with a range of procedures including vaccination, vessel cannulation and minor surgical procedures.\textsuperscript{15-17} It is low cost and is available without prescription in the UK and North America. Its effectiveness has also been demonstrated during the process of ear tattooing in New Zealand white rabbits. Application resulted in less struggling and vocalization, lower peak heart rate and blood pressure and reduced rabbit grimace scale score in comparison to animals that were tattooed without any pain relief.\textsuperscript{18}

The aim of this study was to determine if the MGS is an appropriate technique for assessing pain in mice following ear notching. If so, a secondary aim was to determine the effectiveness of EMLA application to alleviate pain associated with ear notching.

Materials and methods

Baseline image collection

One week prior to notching, the mice were transferred to a quiet room and placed individually in custom-made photography cubes (80 x 80 x 80 mm) that consisted of two clear acrylic sides and two matt white sides. Close-up photographs of the face of the mice were taken across a 10 min period using a high definition camera (Casio EX-ZR100; Casio Computer Co, Ltd, Tokyo, Japan). The mice were photographed on every occasion when they directly faced the camera, apart from when grooming.

Ear notching

Mice were randomly allocated to one of four treatment groups as shown in Table 1. Twenty minutes prior to notching, EMLA (Astra AstraZeneca, Luton, UK) was applied to the ears of the mice. Those mice in the control group underwent the same handing procedure and contact with the ears, but without the application of the EMLA.

At the time of notching, the mice were fully restrained and one ear was notched using a standard ear punch (Vet-tech Solutions Ltd, Cheshire, UK) by a trained and experienced member of the technical staff, who was blind to pre-treatment. Those mice that did not receive an ear notch were restrained in the same manner and the ear punch was clicked in the immediate vicinity of the mouse ear without making any contact.

Post notch image collection

Immediately post notching, the mice were placed into the custom-made photography cubes and the process of image collection was repeated as described above. Images were taken across a maximum period of 5 min.

Image selection and scoring

For each mouse at each time point, three photographs were randomly selected, using a random number generator, from all the clear photographs for future scoring. Each selected photograph was cropped and only

### Table 1. Random allocation of mice to each treatment group.

<table>
<thead>
<tr>
<th></th>
<th>Control no notch</th>
<th>Control notch</th>
<th>EMLA no notch</th>
<th>EMLA notch</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of mice</td>
<td>7</td>
<td>8</td>
<td>7</td>
<td>8</td>
</tr>
</tbody>
</table>

EMLA: eutectic mixture of local anaesthetics.
the head of the mouse remained to prevent any bias in scoring the images due to the posture of the mouse. The selected cropped photographs of each strain were added to pre-designed excel MGS scoring files in a random order, and were assessed by two MGS-trained coders blind to the project aims and methods. For each picture, four facial action units (FAUs), orbit tightening, cheek bulge, nose bulge and ear position were scored based on the MGS method developed by Langford et al.,12 each facial unit was scored on a three-point scale separately (0 = not present, 1 = moderate, 2 = severe), and the sum of all four FAUs were analysed. Whisker position was excluded from the analysis due to the high number of pictures where scorers were unable to assign a score.

**Statistical analysis**

Data were analysed using SPSS software (version 21; SPSS Inc, Chicago, IL, USA). As data collected were not on the continuous scale, they were analysed non-parametrically. MGS scores between the treatment groups (see Table 1) at each time point were compared using a Kruskall–Wallis test. Pre and post ear notch MGS scores were compared within treatment groups using a Wilcoxon test. Results were considered statistically significant when \( P < 0.05. \)

**Results**

The four treatment groups were compared at each time point. There was no significant difference in MGS score between the groups pre or post ear notch (Figure 1). The baseline and post notch MGS scores were compared. There was no significant difference between the two time points.

**Discussion**

Ear notching is a routine husbandry practice used in laboratories to identify individual mice or for genotyping. Based on studies in other species including pigs and rats, the assumption is made that this practice is likely to be painful in mice.5–7 The MGS has been demonstrated to be an effective research tool in pain assessment following a range of procedures where pain is present for between 10 min and 4 h.12 Here, we did not see any change in MGS score immediately following ear notching in male C57BL/6 mice thus indicating that the MGS is not a suitable pain assessment technique for this practice. The data could be interpreted in an alternative way, arguing that no change in MGS score demonstrates that ear notching is not painful in male C57BL/6 mice. However, based on data collected in other species, e.g. rats and pigs5–7 and the suggested limitation with the MGS that it is not effective for...
painful stimuli with a duration of less than 10 min, the assumption can be made that this procedure is likely to be painful unless further evidence can be provided to demonstrate otherwise. Although this finding appears to contrast with change in grimace score observed in rabbits following ear tattooing, this procedure is likely to be more traumatic and so to potentially cause pain over a longer period.

Due to the potential lack of sensitivity of the MGS in this study, potential benefits of applying EMLA prior to notching could not be assessed. However, it is noted that application of EMLA in itself did not result in any changes in the MGS score of the control group, male C57BL/6 mice. Alternative methods of pain assessment should be trialled, for example, focusing on behaviour of the mice immediately following the notching process.

Acknowledgements

The authors would like to thank the animal care staff of the Comparative Biology Centre, Newcastle University for technical assistance and Katherine Tyson for assisting with the data collection.

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References

Assessment and refinement of intra-bone marrow transplantation in mice

U Pfeiffenberger¹, T Yau¹, D Fink¹, A Tichy², R Palme³, M Egerbacher⁴ and T Rülicke¹

Abstract
Intra-bone marrow transplantation (IBMT) may improve the seeding efficiency of transplanted hematopoietic stem cells compared to the routinely used intravenous injection. Current IBMT protocols are optimized for ease of use and to improve experimental results. However, there have been no investigations to assess the impact of IBMT on animal welfare. Here, we report the results of pain assessment after IBMT and the effects of refinements to the current standard procedure. IBMT was performed in either the tibia or the femur of a recipient mouse under general anesthesia. Impact was determined using clinical scoring of different parameters (lameness, grip capacity, body weight loss, footprint analysis), behavioural tests (burrowing, open-field), monitoring of stress hormones and post-mortem histology. The results revealed that IBMT definitely induces severe post-operative distress. Although IBMT in the tibia is technically easier, the degree of impairment and the distress observed were consistently higher than for transplantation in the femur. A refinement for IBMT in the tibia was achieved by using 30- instead of 26-gauge needles and by sparing the patellar tendon. Consequently, for IBMT, we recommend either using the femur, or if the tibia is required due to its better feasibility, using our refined protocol. Furthermore, IBMT should definitely be limited to one leg per animal.

Keywords
intra-bone marrow transplantation, IBMT, mice, refinement

The transplantation of human hematopoietic stem cells into immunodeficient mouse models is traditionally conducted by intravenous (IV) injection into the lateral tail vein. In order to properly proliferate and differentiate, the infused cells have to enter the bone marrow (BM) of the recipient. However, the seeding efficiency of IV transplanted cells in the recipient’s BM to subsequently initiate hematopoiesis is low.¹⁻³ The marginal proportion of transplanted cells which establish in the marrow after entering from the blood (‘homing’) suggests that there is no preferential uptake of cells by the BM. Instead, the infused cells leave circulation shortly after injection and are predominantly found in other organs and tissues.⁴,⁵ This is particularly relevant when only a limited number of donor cells are available. To overcome this problem, intra-bone marrow transplantation (IBMT) has been adapted to mouse models as an alternative method of stem or progenitor cell transplantation.⁶ Since the cells are deposited directly into the recipient’s BM, it has a higher rate of donor-cell engraftment compared to the IV transplantation route.⁷,⁸

During the last decade, murine IBMT has been applied in studies ranging from stem cell therapy to cancer research.⁹⁻¹⁶ Intraosseous transplantation is routinely conducted in the tibial shaft of the mouse after a small incision on the thigh. To reach the bone cavity, a hole for the microsyringe is drilled by inserting a 26- or 27-gauge needle into the joint surface of the tibia through the patellar tendon. The experimental procedure is performed under general anesthesia. Transplantations in the femur have also been described but are rare in comparison to tibia injection.¹⁷,¹⁸

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To our knowledge, there has not yet been a comprehensive analysis of the impact of IBMT on animal welfare. However, this surgery could potentially result in considerable post-operative pain and distress. Therefore, the aim of this study was to evaluate the actual impact of standard IBMT in the tibia of mice. In addition, we determined the effects of using the femur as an alternative injection site and refined the standard procedure to reduce distress in mice from IBMT.

Materials and methods

Animals

Female BALB/cAnNCrl mice (7–8 weeks old; Charles River, Sulzfeld, Germany) were housed in groups of 6–8 per type 3 Makrolon cage (Tecniplast, Buguggiate, Italy) under standard housing conditions (room temperature 21±1°C, relative humidity 50±10%, artificial 12h light–dark cycle, lights on from 07:00h to 19:00h). Access to food (V1126; Ssniff Spezialitäten GmbH, Soest, Germany) and tap water were ad libitum. Cages were lined with bedding material (Lignocel®, heat treated; Rettenmaier & Söhne GmbH, Rosenberg, Germany) and enriched with a mouse house, two board tubes and nesting material (Pur-Zellin; Paul Hartmann AG, Heidenheim, Germany). The adaptation period before the experiment was 10 days. During the training period and the first three days post-operation, the mice were housed individually in type 3 Makrolon cages equipped with the burrowing set-up. All tests were carried out during the light phase. Animal number and treatment groups are summarized in Table 1.

The distress induced by the IBMT was prospectively categorized as ‘medium’. The study was approved by the institutional ethics committee of the University of Veterinary Medicine Vienna, and an experiment license was granted under BMWF-68.205/0183-II/3b/2011 (Austrian Federal Ministry of Science and Research).

Table 1. Number of animals in different treatment and control groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Anesthesia</th>
<th>Pain relief</th>
<th>Site of injection</th>
<th>Treatment</th>
<th>No. of animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Yes</td>
<td>Days 0–3 Tibia</td>
<td>Standard</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Yes</td>
<td>Day 0 Femur</td>
<td>Standard</td>
<td>24</td>
<td></td>
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<tr>
<td>3</td>
<td>Yes</td>
<td>Days 0–3 Tibia</td>
<td>Refined</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Yes</td>
<td>Day 0 Femur</td>
<td>Refined</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Yes</td>
<td>Day 0 – –</td>
<td>–</td>
<td>23</td>
<td></td>
</tr>
</tbody>
</table>

Experimental design

Prior to the operations, the mice were trained to perform the burrowing test and the footprint analysis over a five-day period. After the training period, they had two days to recover. The baselines for stress hormones, burrowing activity and footprint analysis were determined prior to the IBMT. To avoid the impact of circadian rhythm, all daily tests and measurements were carried out at the same time of day. The schedule of all treatments and assessments performed for each animal during the project is summarized in Table 2.

Table 2. Schedule for the experimental procedures and tests before and after intra-bone marrow transplantation (IBMT).

<table>
<thead>
<tr>
<th>Day</th>
<th>Time</th>
<th>Action</th>
<th>Test performed</th>
</tr>
</thead>
<tbody>
<tr>
<td>−8</td>
<td>08:00 h</td>
<td>Single housing</td>
<td>General health assessment</td>
</tr>
<tr>
<td></td>
<td>10:30 h</td>
<td>Training</td>
<td>Burrowing</td>
</tr>
<tr>
<td></td>
<td>13:00 h</td>
<td>Training</td>
<td>Footprint</td>
</tr>
<tr>
<td>−7</td>
<td>08:00 h</td>
<td>Training</td>
<td>Burrowing</td>
</tr>
<tr>
<td></td>
<td>10:30 h</td>
<td>Training</td>
<td>Burrowing</td>
</tr>
<tr>
<td></td>
<td>13:00 h</td>
<td>Training</td>
<td>Footprint</td>
</tr>
<tr>
<td>−6</td>
<td>08:00 h</td>
<td>Training</td>
<td>Burrowing</td>
</tr>
<tr>
<td></td>
<td>10:30 h</td>
<td>Training</td>
<td>Burrowing</td>
</tr>
<tr>
<td></td>
<td>13:00 h</td>
<td>Training</td>
<td>Footprint</td>
</tr>
<tr>
<td>−5</td>
<td>08:00 h</td>
<td>Training</td>
<td>Burrowing</td>
</tr>
<tr>
<td></td>
<td>10:30 h</td>
<td>Training</td>
<td>Burrowing</td>
</tr>
<tr>
<td></td>
<td>13:00 h</td>
<td>Training</td>
<td>Footprint</td>
</tr>
<tr>
<td>−4</td>
<td>08:00 h</td>
<td>Pre-op measurement</td>
<td>Burrowing</td>
</tr>
<tr>
<td></td>
<td>11:00 h</td>
<td>Pre-op measurement</td>
<td>Footprint</td>
</tr>
<tr>
<td>−3</td>
<td>Recovery</td>
<td></td>
<td></td>
</tr>
<tr>
<td>−2</td>
<td>Recovery</td>
<td></td>
<td></td>
</tr>
<tr>
<td>−1</td>
<td>09:00 h</td>
<td>Pre-op measurement</td>
<td>Clinical scoring</td>
</tr>
<tr>
<td></td>
<td>10:00 h</td>
<td>Post-op measurement</td>
<td>Stress hormones (Baseline value)</td>
</tr>
<tr>
<td></td>
<td>12:00 h</td>
<td>Pre-op measurement</td>
<td>Footprint</td>
</tr>
<tr>
<td>0</td>
<td>12:00 h</td>
<td>IBMT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15:00 h</td>
<td>Post-op measurement</td>
<td>Footprint</td>
</tr>
<tr>
<td></td>
<td>16:00 h</td>
<td>Post-op measurement</td>
<td>Burrowing</td>
</tr>
<tr>
<td>1</td>
<td>09:00 h</td>
<td>Post-op measurement</td>
<td>Clinical scoring</td>
</tr>
<tr>
<td></td>
<td>10:00 h</td>
<td>Post-op measurement</td>
<td>Stress hormones</td>
</tr>
<tr>
<td></td>
<td>12:00 h</td>
<td>Post-op measurement</td>
<td>Open field</td>
</tr>
<tr>
<td></td>
<td>13:00 h</td>
<td>Post-op measurement</td>
<td>Footprint</td>
</tr>
<tr>
<td>2</td>
<td>09:00 h</td>
<td>Post-op measurement</td>
<td>Clinical scoring</td>
</tr>
<tr>
<td></td>
<td>10:00 h</td>
<td>Post-op measurement</td>
<td>Stress hormones</td>
</tr>
<tr>
<td>3</td>
<td>09:00 h</td>
<td>Post-op measurement</td>
<td>Clinical scoring</td>
</tr>
<tr>
<td></td>
<td>10:00 h</td>
<td>Post-op examination</td>
<td>Stress hormones</td>
</tr>
</tbody>
</table>

Table 2. Schedule for the experimental procedures and tests before and after intra-bone marrow transplantation (IBMT).
Anesthesia and analgesia

For IBMT, the animals received a combined anesthesia of midazolam (0.7 mg/kg, Dormicum®; Roche, Vienna, Austria), medetomidine (7 mg/kg, Domitor®; Pfizer, Vienna, Austria), and fentanyl (0.07 mg/kg, Fentanyl-Janssen®; Janssen-Cilag Pharma GmbH, Vienna, Austria) (MMF) via intraperitoneal (IP) injection. Before the IBMT was performed, the surgical tolerance was tested by the toe-pinch withdrawal reflex. The anesthesia was antagonized after 40 min with a subcutaneous (SC) injection of a mixture of atipamezole (2.6 mg/kg, Antisedan®; Pfizer) and flumazenil (0.53 mg/kg, Anexate®; Roche). In order to keep the analgesic effect of fentanyl, its antagonist naloxone was replaced by 0.9% NaCl (Fresenius Kabi, Graz, Austria). In addition, buprenorphine (0.1 mg/kg, Temgesic®; AESCA Traiskirchen, Austria) was administered SC during IBMT. In order to assess the real impact of the treatment and to avoid side-effects from analgesic drugs, we originally intended to conduct the IBMT without further pain relief. However, during a pilot study it became obvious that the animals suffered significantly from post-operative pain for an extended time. Therefore, for ethical reasons, a second injection of buprenorphine was given 3 h post-IBMT to all animals. To avoid affecting the stress and pain indicators, additional analgesia was avoided until the footprints analysis, open-field test, stress hormone, and burrowing tests were complete (day 3). However, it appeared to be unethical to allow the animals to be in pain for the time necessary to complete the clinical scoring so those that still had a high clinical score after performing the post-operation measurements (both tibia transplantation groups), received an additional administration of buprenorphine on day 3.

Surgical procedure

Anesthetized mice were placed on a warming plate to prevent hypothermia. Eyes were protected with eye ointment (Oleovit; Fresenius Kabi). In contrast to the method described in the literature, we generally avoided making an incision on the thigh. For better visualization of the anatomical structures, the region around the knee was depilated with cream (Veet; Reckitt Benckiser AG, Wallisellen, Switzerland) before disinfection with 70% ethanol (EtOH). Only one leg (the right) was treated per animal.

For the standard IBMT in the tibia, the knee was flexed at about 90° and a 26G × 12 mm needle was inserted into the joint surface through the patellar tendon and drilled into the BM cavity. The refined method for the IBMT was conducted in the same way except with a 30G × 12 mm needle and avoided the patellar tendon (Figure 1a). In both procedures, the needle was removed and a microsyringe (10 μL; Bartelt GmbH, Graz, Austria, RN, 30G × 12 mm) was introduced into the drilled tunnel to inject 5 μL human lymphocyte culture medium (RPMI 1640 medium, PAA, Cat. No. E15-842) into the BM cavity (Figure 1b). The transplantation into the medullary cavity of the distal femur was conducted in a similar manner as described for the tibia. However, due to anatomical reasons it was not possible to avoid a patellar tendon lesion. Therefore, the refined method for the femur transplantation differed from the standard procedure only in the reduction of the size of the needle used to drill the bone hole (Figure 1c).

Clinical scoring

The clinical scoring parameters and the corresponding scoring scheme are summarized in Table 3. For the observations of lameness, the animals were placed in an empty cage. The ‘lameness score’ parameter was estimated mainly from limping, posture during movement, gait coordination and exploration activity.

The grip capacity was assessed by placing the mouse with its hind legs on the edge of the home cage, and the mouse’s ability to grip the edge of the cage with the injected leg was noted. This was done three times in a row for each mouse and the results were averaged.

Body weight was measured for seven days post-operation using an electronic scale.

The set-up for footprint analysis consisted of a runway of 60 cm, guiding the mice from a light tunnel through a dark tunnel into a mouse house, similar to that from their home cage. Sunflower seeds in the house additionally motivated the animals to take the runway. To record the footprints of the mice, two paper sheets (DIN A4) were placed along the runway. The hind paws of the mice were painted with non-toxic blue ink immediately before their run. Gait analysis was carried out by measurement of the step length and step width of the footprints.19 In addition, post-operative changes such as sharpening of footsteps, toe position, balance, compensation steps and rhythmic/linear movement were noted.

Behavioural scoring

The burrowing test was performed according to Deacon and Jirkof et al. with minor modifications.20,22 In brief, two standard water bottles (250 mL), one filled with food pellets and one left empty, were provided in combination with nesting material in the home cage. During the experiment, burrowing was visually observed for a maximum of 30 min after each morning refill. Burrowing behaviour was defined as the spontaneous removal of more than five pellets in 10 s from
the burrowing tube without the need to feed, to hoard or to hide. The latency to burrow was not considered. In addition, overnight burrowing was recorded in the morning of days 0, 1, 2 and 3.

In order to measure general locomotor activity and willingness to explore, the open-field test was applied on day 1 post-operation. Individual mice were placed in the centre of a square arena (40 cm × 40 cm × 60 cm) and were allowed to explore the open field freely for 5 min. Standard parameters for locomotor activity (total distance, average speed, resting time, transits through the centre and time spent on the edges) were recorded with the automated video-computational TiBeSplit1.0 tracking system (Sony Color Video Camera CVX-V18NSP).

**Monitoring of stress hormones**

Concentrations of corticosterone metabolites were measured in the feces of the animals before (baseline

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**Figure 1.** [a] For intra-bone marrow transplantation (IBMT) the needle was inserted into the joint surface and drilled into the bone marrow cavity of the tibia [refined method]. [b] Introduction of the microsyringe into the drilled tunnel for IBMT in the tibia. [c] Inserted needle in the bone marrow cavity of the femur after drilling through the joint surface [standard method].

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**Table 3. Clinical scoring scheme.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lameness score</td>
<td>No lameness</td>
<td>Slightly lame, protective pose</td>
<td>Medium lameness, tip-toe walk</td>
<td>Highly lame, no use of the operated leg</td>
</tr>
<tr>
<td>Grip capacity of injected leg</td>
<td>Normal</td>
<td>Slightly reduced</td>
<td>Moderately reduced</td>
<td>No grip</td>
</tr>
<tr>
<td>Body weight loss</td>
<td>&lt;5%</td>
<td>&lt;10%</td>
<td>&lt;20%</td>
<td>≥20%</td>
</tr>
</tbody>
</table>
value) and after IBMT. Fresh feces were collected while the animals were housed individually in an empty cage for one hour. The lag time for corticosterone metabolites in feces was estimated to be about 8–10 h. Therefore, fecal collection was scheduled in the morning to avoid any impact from the other tests performed the day before. Samples were placed immediately on ice and stored at −18°C. For the analysis, fecal samples were homogenized, and 0.05 g was mixed with 1 mL of 80% methanol. The samples were vortexed, centrifuged, and the supernatant was diluted 1:20 with assay buffer. Concentrations of corticosterone metabolites were analyzed by an in-house 5α-pregnane-3β,11β,21-triol-20-one enzyme immunoassay (EIA). For further details of the EIA and cross-reactivity of the antibody see Touma et al.23,24

Histological examination

After completion of the tests, the animals with the lowest and the highest clinical scores in each treatment group were sacrificed by cervical dislocation and the treated (right) hind legs were dissected. In order to examine the immediate post-operative impact of IBMT, age-matched BALB/c females were sacrificed and a standard IBMT was performed in either the femur or the tibia of one hind leg. Afterwards, treated and untreated legs were isolated for histological examination. The collected specimens were immediately fixed in 4% buffered formaldehyde solution, decalcified in 8% EDTA and subsequently embedded in paraffin. Serial sections were stained with hematoxylin & eosin (H&E).

Statistics

Results are expressed as means. Differences were assessed by repeated measurement and one-way analysis of variance (ANOVA) with Dunnet’s post-hoc procedure. If ANOVA was not appropriate, Kruskal–Wallis test, Mann–Whitney U-test and Wilcoxon W-test were used. Also, Spearman’s rank correlation coefficient, Pearson’s correlation coefficient and Pearson’s chi-square test were used to detect correlations among results. Data were analyzed using IBM SPSS v19.0 (SPSS Inc, Chicago, IL, USA), and a P value of <5% (P < 0.05) was considered to be significant.

Results

Clinical scoring

The clinical scoring clearly demonstrates the adverse effects of IBMT on the mice for all measured parameters, independent of the transplantation site and the refinement of the procedure (Figures 2a, 2b and 2c). See also Supplementary Figures S1a, S1b and S2 (all supplementary materials can be found online with this article at http://lan.sagepub.com).

The degree of lameness and the reduction of grip capacity were more distinct for tibia groups compared to the femur groups, suggesting a higher and more prolonged burden after tibia injection (Figures 2a and 2b). There were significant differences in lameness scores between the femur groups and the controls until day 8 (standard method, P = 0.008) and day 9 (refined method, P = 0.015) post-operation whereas animals after standard tibia injection remained significantly different until day 20 post-operation (P = 0.009). The refinement of the injection technique was most effective for tibia injection, resulting in a generally lower degree of lameness and recovery on day 16 post-operation, four days earlier than with the standard method.

A similar impact of IBMT was observed regarding the grip capacity of the treated leg. The femur groups recovered on days 9 and 10 post-operation, respectively. Although both tibia groups also exhibited continuous improvement, the grip capacity remained significantly impaired until day 17 of the observation period for the standard method (P = 0.027) and until day 21 post-operation for the refined method (P = 0.021) (Figure 2b). Boxplots for lameness and grip capacity reveal a reciprocal development of both parameters over time (Supplementary Figures S1a and S1b). The lameness decreased and the grip capacity improved continuously over the post-operative observation period. This was confirmed by strong negative Spearman rank correlation coefficients from r = −0.66 to −0.78 between lameness and grip capacity for the post-operative observation period.

All experimental groups (including the control groups) reacted to the anesthesia with a body weight loss of 4–8% on day 1 post-operation (Figure 2c). With the exception of the standard tibia group, the other animals needed about six days to gain back their pre-operative body weight. Although the animals of the refined tibia group still exhibited a significant reduction in body weight on day 3 post-operation (P = 0.041), their body weights then rapidly increased to pre-operative levels. By contrast, the body weights of the standard tibia group were still significantly lower (P = 0.011) than the controls even seven days post-operation (Figure 2c).

Scoring results for lameness and grip capacity were supported by observed changes in the footprint profile for injected mice (Supplementary Figure S2). On day 1 post-operation we observed nonlinear movement, compensation steps of the untreated leg, and a lack of definition of the footprints. Measurement of step lengths and step widths revealed no significant
differences between experimental groups and controls (data not shown).

**Behavioural scoring**

To assess any distress or discomfort due to IBMT, we also investigated spontaneous burrowing behaviour. All mice burrowed rigorously on day −1 (pre-operation). About 3 h post-operation, the burrowing behaviour was reduced in all groups, suggesting the antagonized anesthesia continued to have a strong effect on the animals at this time point. There were no significant differences between any experimental group and the controls on day 0 and day 1. However, 24 h post-operation, burrowing of both refined groups returned nearly to their pre-operative levels whereas post-operative burrowing was at the lowest level (not significant) in the tibia group injected using the standard method (Figure 3). All animals but one (from the standard tibia group) engaged in overnight burrowing even during the first night after surgery (data not shown).

The open-field test was conducted on day 1 post-operation. No significant differences to the control group were observed in any measured parameter in both femur IBMT groups. The tibia groups did not differ in the transits through the center and time spent on the edges (data not shown). However, the distances travelled were significantly reduced in both the standard tibia group ($P = 0.027$) and the refined tibia group ($P = 0.041$) (Supplementary Figure S3). Furthermore, we observed a decreased speed in both tibia groups which was significant in animals from the refined tibia group ($P = 0.036$; Supplementary Figure S4).

**Figure 2.** Results of the clinical scoring. (a) Lameness scores of animals in different treatment groups from day 1 to day 21 after intra-bone marrow transplantation (IBMT). (b) Grip capacity scores of animals in different treatment groups from day 1 to day 21 after IBMT. (c) Body weight of animals in different groups from day 1 to day 7 after treatment.
Monitoring of stress hormones

To assess the pain and suffering of the animals after IBMT, we monitored stress hormone metabolites in the feces. There was a significant increase of stress hormones for the first three days post-operation in all groups including the controls. A significant increase of corticosterone metabolites compared to the controls was only detected for the standard tibia group on day 3 post-operation ($P=0.03$; Figure 4).

Histology

Figure 5a shows a histological section through the knee joint of a control animal depicting femur, tibia and the cruciate ligaments in the center of the joint cavity. The section through the knee joint of directly treated animals clearly shows the drilled channel penetrating the epiphysis and growth plate, reaching the BM cavity of the diaphysis (Figures 5b and 5c). Remnants of the previous penetration were visible as bony formations within the growth plate three weeks after treatment (Figures 5d and 5e). In addition, severe connective tissue proliferation and scar formation were repeatedly detected in animals treated by the standard tibia method (Figure 5d) but not by the femur route despite penetrating the patellar tendon. In the refined tibia group, connective tissue proliferation was found for one animal with a high clinical score; however it was much less intense than in the standard treatment (data not shown).

Discussion

IBMT in mouse models is a method for delivering (malignant) hematopoietic stem cells directly to their preferred engraftment site. It has been shown that the BM microenvironment promotes the survival and proliferation of the transplanted cells. The presented study was conducted to determine the distress induced by IBMT in the tibia and the femur of laboratory mice. Clinical scoring, behavioural tests, measurement of corticosterone metabolites and post-mortem histology revealed a significant impact on the well-being of treated animals. This finding was generally more pronounced for transplantation into the tibia compared to the femur. The adopted refinement of tibia injection by using smaller injection needles and avoiding the patellar tendon resulted in a measurable reduction of the impairment and accelerated recovery of treated animals compared to the standard method (except grip capacity, Figure 2b).

IBMT in mice is currently routinely conducted in the tibia. Due to the larger muscles in the thigh, the femur

![Figure 3. Scoring results for burrowing behavior of different experimental groups and the controls before and after treatment.](image_url)
is poorly accessible for fixation, making IBMT more difficult for the experimenter. However, a possible advantage of the femur is that the condyles can serve as a guiding structure for the cannula into the BM cavity. In addition, in a flexed position, the anterior and posterior cruciate ligaments will not be encountered upon penetration. The higher burden seen after tibia injection coincides with severe connective tissue proliferation and scar formation around the cruciate ligaments (Figure 5d) since they cannot be avoided when injecting into the tibia. This malformation was mostly seen in animals after standard IBMT in the tibia but not found after femur injection. However, in the refined tibia group, connective tissue proliferation was observed at a lower degree and only in an animal with a bad clinical score (data not shown).

Pain signs such as increased lameness, decreased grip capacity and abnormal walking patterns occurred in all groups with IBMT. Furthermore, we found destroyed nests in the morning of day 1 even though all the mice were highly motivated pre-operation to build a nest. Nest destruction was observed in both experimental groups and the controls, suggesting a strong impact of the MMF anesthesia on the well-being and behavior of the animals during the first two days post-operation. The observations coincided with the results of the body weight and the burrowing test. Both seemed to be partially superimposed by the anesthesia since the control group exhibited similar changes to animals of the IBMT groups over the first days post-operation (Figures 2c and 3). Therefore, the results of body weight and behavioural scoring during the early post-operative period were obviously biased for the assessment of the level of discomfort and harm inflicted by IBMT.

In contrast to lameness and grip capacity, whose slow recovery reflected the impairment of the affected limbs, burrowing soon recovered to pre-operative levels. Good performance of spontaneous home cage behaviours like burrowing is thought to be indicative of well-being. Assuming that home cage behaviours are negatively affected in suffering mice, the fast resumption of burrowing after IBMT suggests that mice can cope with the impairment of one affected limb. Furthermore, the rapid return to pre-operative levels of this self-rewarding behaviour suggests no high and lasting levels of pain and distress after IBMT, despite the lingering of the disability.

Stress hormones were analyzed in the feces of treated animals and controls (Figure 4). Although the animals were undisturbed for two days before collecting feces,

**Figure 4.** Boxplots for corticosterone metabolites measured in the feces of different experimental groups and controls before and after treatment. Medians (lines in boxes), 25–75% interquartile range (iqr) (boxes), range from smallest to highest value excluding extreme values (whiskers), extreme values more than 1.5 times iqr above q3 or below q1 (●) or more than 3 times iqr above q3 (*). IBMT: intra-bone marrow transplantation.
Figure 5. Histological section through the knee joint of a [a] control animal depicting femur [F], tibia [T] and the cruciate ligaments [CL] in the centre of the joint cavity. The knee joint of animals directly injected with the standard method in [b] the tibia and [c] the femur clearly shows a channel penetrating the epiphysial and growth plate and reaching the bone marrow cavity of the diaphysis [black arrow]. Section through the knee joint of an animal three weeks after treatment with the standard method in [d] the tibia and with the refined method in [e] the femur demonstrating remnants of the previous penetration as bony formations within the growth plate [arrow]. In addition, severe connective tissue proliferation and scar formation [asterisk] is seen at the site of the CL after standard tibia injection [d]. Staining with hematoxylin & eosin (H&E), scale bar = 300 μm.
for the pre-operative measurement, the overall baseline for corticosterone metabolites determined at day —1 was generally higher, as could have been expected from our previous measurement data. As we can exclude external stressors, we assume that this is a residual impact from the prior five-day training period.

The levels of stress hormone metabolites increased significantly after IBMT in all treatment groups and in the control group, revealing that, post-operation, MMF anesthesia also has an effect on stress hormones that was obviously not compensated by the analgesic treatment. A significant increase in levels of stress hormone metabolites compared to those in the non-injected controls was only observable on day 3 for the standard tibia group.

In conclusion, the presented study demonstrates that IBMT in the tibia and the femur of mice has a significant impact on the well-being of the animals. Different tests have revealed that the technically simpler, and thus more common, tibia injection is more harmful to the animals, probably due to the lesions in the cruciate ligaments of the knee followed by connective tissue proliferation and scar formation. Thus, the femur should be the favored IBMT injection site. However, if this is not possible we strongly recommend a refinement by reducing the size of the injection needle and by sparing the patellar tendon. Because the treated limb cannot be used for up to three weeks, IBMT should definitely be limited to one leg per animal, irrespective of the bone chosen.

Acknowledgements

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Declaration of conflicting interests

None declared.

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References

16. Shi M, Li M, Cui Y, Adachi Y and Ikehara S. Gr-1 Ab administered after bone marrow transplantation plus thymus transplantation suppresses tumor growth by


Comparison of haematopoietic stem cell engraftment through the retro-orbital venous sinus and the lateral vein: alternative routes for bone marrow transplantation in mice

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Abstract
Bone marrow transplantation in mice is performed by intravenous administration of haematopoietic repopulating cells, usually via the lateral tail vein. This technique can be technically challenging to carry out and may cause distress to the mice. The retro-orbital sinus is a large area where there is a confluence of several vessels that provides an alternative route for intravenous access. Retro-orbital injection, although aesthetically unpleasant, can be performed rapidly without requiring mechanical restriction or heat-induced vasodilation. In addition, this technique can be easily learned by novice manipulators. This route of administration has been reported for use in bone marrow transplantation but there is no comparison of retro-orbital and tail vein injections reported for this specific purpose, although both routes have been compared for many other applications. Here, we provide for the first time a comprehensive comparison between tail vein and retro-orbital injections for two different bone marrow transplant scenarios in P3B and B6D2F1 mice. In both cases, no significant differences regarding donor engraftment were observed between mice transplanted using each of the techniques. Haematological counts and leukocyte subpopulation distribution were practically identical between both animal groups. Moreover, donor engraftment levels were less homogenous when cells were transplanted by tail vein injection, probably due to a higher risk of failure associated with this technique. All these data suggest that retro-orbital injection is a compelling alternative to conventional tail vein injection for bone marrow transplant in mice, providing similar and more homogenous haematopoietic reconstitution.

Keywords
bone marrow transplant, intravenous injection, retro-orbital sinus

The mouse, *Mus musculus*, is the most commonly used laboratory animal. One of the most common and efficient routes of administration or injection in mice is the intravenous (IV) route.1 It can be used to administer cells, chemical agents, infectious disease agents, drugs and any other reagent that needs a systemic delivery.

IV vascular access is technically challenging in the adult mouse. The most common technique for IV delivery is tail vein (TV) injection, which requires practice and accuracy to be correctly performed. Mice have two...
superficial lateral tail veins, and a single artery along the ventral side of the tail. Intra-arterial administration is usually avoided because of potentially severe complications including blindness, cerebrovascular stroke, permanent motor deficits, and limb gangrene. The lateral tail veins are therefore the route of choice in most situations. These veins are thin and prior warming up of the mouse is required to promote vasodilation. A very useful but not very popular alternative to this procedure is injection in the retro-orbital (RO) venous sinus of the mouse. The RO sinus is a large area with a substantial amount of blood flow in which several vessels meet, including the supraorbital vein, inferior palpebral vein, dorsal nasal vein and the superficial temporal veins. An accurate description of the RO sinus injection technique in mice was lacking until a very detailed work was published in 2011. In addition, some authors have compared these two routes in some specific situations. It has been demonstrated that the RO route can be used equivalently to the TV route for IV administration of monoclonal antibodies, cells and chemical agents. The authors did not find any differences, either in organ distribution or blood decay profiles, between the two groups. Moreover, in some cases the RO route has appeared to be superior to the TV route. Socher et al. found that the RO administration of contrast media for cardiac perfusion studies in mice using computed tomography provided better cardiac imaging results than TV injections.

Some researchers have also claimed that RO administration causes less stress to the animals, although it was not until 2008 that Steel et al. published some data to support this. The authors performed a direct comparison of TV and RO injections as IV drug delivery routes in mice. Although no differences were observed in the effects caused by the administered drug between the two routes, they found that, at least in BALB/c mouse strain, injection via the RO route was much less stressful than via the TV. In addition, they measured stress-related parameters, finding much lower stress-related scores in RO-injected mice than in TV-injected mice.

However, apart from the works mentioned above, there is still very little information about particular procedures using this route in the literature. One of these particular procedures is bone marrow transplantation (BMT).

The haematopoietic system follows a hierarchical organization in which haematopoietic stem cells (HSCs) are at the top of the pyramid. They are minimally present in the bone marrow (BM) and are characterized by their ability to generate the various cell types in the blood and by their self-renewal capability. Mouse haematopoietic progenitors are defined as a heterogeneous population with short- and long-term repopulation ability that do not express the typical lineage markers expressed by mature blood cells, so they can be defined as lineage negative (Lin) cells. This Lin− cell fraction contains a population of cells expressing the Sca1 and c-kit markers known as Lin−, Sca1+ and c-Kit+ (LSK) cells, which correspond to the most primitive mouse HSCs.

BMT is a routine technique applied when studying the haematopoietic system. It involves the IV administration of haematopoietic repopulating cells into specific recipients. Interest in BMT goes beyond the basic knowledge of the haematopoietic system as it can be a therapeutic option for many haematopoietic and non-haematopoietic diseases. BMT in mice is usually performed by TV injection, and no comparisons of TV and RO routes have been published for this particular application. The only published data, to our knowledge, was produced in the context of xenotransplantation and conditioning studies for HSC transplantation. The authors found that limiting numbers of human HSCs could be transplanted by the RO route, reaching higher human chimerism levels than when an equal number of cells were transplanted by the TV route. There are also some data comparing intra-femoral and RO administration of HSCs in the context of syngeneic BMT in mice, demonstrating that these two routes are also equally efficient.

Many authors would find it interesting to have another choice other than IV injection, but the lack of expertise and information in the literature about the actual rate of success and engraftment using the RO route for BMT might deter them. Evidence is still needed to determine whether the route of administration might have an effect on HSC engraftment or in different multilineage reconstitution. Thus, we present here an accurate comparison between TV and RO routes for BMT in mice. We demonstrate that RO injections can be superior in some circumstances and that, on balance, both routes can be considered as equivalent for BMT.

Materials and methods

Animals

B6.SJL-Ptpcrea/bPep3b/BoyJ (P3B10); B6D2F1 and (B6.SJL-Ptpcrea/bPep3b/BoyJx-DBA/2) F1 (P3D2F1) colony founders were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and bred in-house for a maximum of five generations at the CIEMAT Animal Facility, Madrid, Spain (registration number ES280790000183).

The vavHS21/45ΔhCD4 (vavΔhCD4) transgenic mice were previously generated in the P3B strain (CD45.1+) in our laboratory. These mice express a
human CD4 truncated protein (ΔhCD4) which is incapable of intracellular signalling or of association with the major histocompatibility complex (MHC) class II in all cells of the haematopoietic system.

The mice were maintained under high standard conditions and were routinely screened for pathogens in line with Federation of European Laboratory Animal Science Associations (FELASA) recommendations for the health monitoring of mouse colonies in breeding and experimental units. The animals were maintained with HEPA-filtered air with an exchange rate of 16–20 changes per hour, and with regulated temperature of 20 ± 2°C and humidity of 55 ± 10%. An artificial light/dark cycle of 13/11 h was followed with lights on at 07:00 h. They were fed a 20 Gy-irradiated standard diet (Teklad Global Diets 2914; Harlan Laboratories Inc, Indianapolis, IN, USA) and water ad libitum. The water was obtained from the human supply network, filtered through 5 microns, ultraviolet-irradiated, acidified at pH 2.5 and autoclaved. Dust-free and heat-sterilized soft wood pellets from the white poplar (Populus alba) was used as bedding (Souralit, Girona, Spain). The animals were housed five to six mice per group in polyphenylsulfone cages with a 435 cm² area (Type 1145T; Tecniplast, Buguggiate, Italy) enriched with cellulose non-woven blocks (Nestlets; Bioscape, Castrop-Rauxel, Germany) and paper wool (Souralit, Girona, Spain).

All experimental procedures were carried out according to Spanish and European regulations (Spanish Royal Decree 53/2013 and Law 6/2013, which translate and comply with the European Directive 2010/63/UE about the use and protection of vertebrate mammals used for experimentation and other scientific purposes). At the end of the experimentation all animals were euthanized using carbon dioxide.

**BMT studies**

Two different BMT scenarios were used for the comparison of TV and RO routes. In the first study, 10 P3B female recipients (8 week old) with a mean body mass of 22 ± 3 g were lethally irradiated with two doses of 4.75 Gy spaced 24 h apart and were then transplanted with 3 × 10⁶ total bone marrow (TBM) cells obtained from three 6-week-old vavΔhCD4 male mice with a mean body mass of 21 ± 3 g. Five mice were transplanted by RO injection (experimental group) and the other five by TV injection (control group). Peripheral blood (PB) was collected monthly and analysed for donor engraftment levels (determined by flow activated cell sorting [FACS] as the percentage of hCD4⁺ cells). multilineage distribution was also determined by FACS as the percentage of B220⁺, CD3⁺ and Gr-1⁺/CD11b⁺ cells at three months post transplantation (mpt). Haematological counts were determined at 1 and 3 mpt. At 3 mpt, mice were sacrificed and BM cells were collected and analysed for donor engraftment in the total BM population and inside the LSK subpopulation.

In the second study, ten female B6D2F1 mice (8 week old) with a mean body mass of 24 ± 3 g were sub-lethally irradiated with one dose of 5 Gy and 24 h later they were transplanted with 1500 FACS-sorted LSK cells. These cells were obtained from three 6-week-old female P3D2F1 mice with a mean body mass of 25 ± 3 g. Five mice were transplanted by RO injection (experimental group) and the other five by TV injection (control group). The same analyses and the same timeline were applied here as the ones described for the first study; with the exception of donor chimerism level determination, which was performed on the basis of the percentage of CD45.1⁺ cells.

The irradiation conditioning was carried out using an X-ray equipment MG324 (300 kV, 12.8 mA; Philips, Hamburg, Germany) placed in the Animal Facility.

**TV injections**

TBM cells or FACS-purified LSK cells were IV transplanted through lateral TV injection. The mice were placed under a heat lamp to promote peripheral vaso-dilation and were then mechanically restrained using a home-made Plexiglas chamber. The tail was then kept horizontally straight to allow the manipulator to perform the injection. Syringes with 25-gauge needles were used for the TV administration of the cells. A maximum volume of 200 µL was injected. After manipulation the animals were returned to their cages for recovery.

**Retro-orbital venous sinus injections**

TBM cells or purified LSK cells were IV transplanted through RO sinus injection. The mice were anaesthetized using continuous inhalation of isoflurane (IsoFlo, Abbott Laboratories Ltd, Queenborough, Kent, UK; 4–5% as the induction dose and 1.5–3% as the maintenance dose in one LPM medicinal oxygen as the carrier gas) and a Plexiglas induction chamber. When the required depth of anaesthesia was reached, as indicated by the absence of the corneal reflex and toe pinch, the mouse was taken out of the chamber and placed in a left lateral recumbency position with its head facing to the right. This position was optimal for right-handed manipulators. Then, gentle pressure was applied to the right eye socket to partially protrude the mouse’s eyeball. A 31-gauge needle was carefully introduced, bevel down, at an angle of approximately 30°. A maximum
volume of 200 μL was injected. When the needle was withdrawn, usually no bleeding was observed. When the injection was complete the mice were placed on a heating pad to ensure body temperatures of about 34°C, and were then placed back into their cages for recovery.

**PB collection**

PB collection was carried out monthly after transplantation as well as at the end of the experiments when the animals were culled. PB was mixed with 20 μL of ethylenediaminetetraacetic acid (EDTA) to prevent blood coagulation. For the PB periodical collection, mice were placed under a heat lamp to promote peripheral vasodilation and were then mechanically restrained using a home-made Plexiglas chamber. Then, after local administration of anaesthetic (EMLA [lidocaine/prilocaine]; AstraZeneca, Madrid, Spain), a small transverse cut was performed in the lateral TV with a sterile blade. Blood drops were collected with a micro-pipette and mixed with EDTA. A maximum volume of 100 μL of blood was obtained. After blood collection a slight pressure was applied to ensure that the bleeding would stop and the mice were put back into their cages for recovery.

When the animals were culled at the end of the experiment, PB was collected by cardiac puncture. Immediately after euthanization with carbon dioxide, the animals were placed in a right lateral recumbency position. The abdomen skin was moistened with 70% alcohol and then cut to visualize the abdomen wall. A one-millilitre syringe with a 25-gauge needle was slowly and perpendicularly inserted between ribs 5 and 6 and close to the sternum. After blood collection the needle was carefully withdrawn and cervical dislocation was performed to ensure that the animal was deceased. A maximum volume of 800 μL of blood was obtained.

**BM cell collection**

For the collection of the BM cells, mice were sacrificed and both tibiae and femora were surgically extracted. BM was harvested by flushing the tibiae and femora under sterile conditions with Dulbecco’s modified Eagle medium + GlutaMAX (DMEM; Gibco/Life Technologies/Thermo Fisher Scientific, Waltham, MA, USA) and washed twice with phosphate buffered saline (PBS; Sigma-Aldrich, St Louis, MO, USA).

**Haematological counts**

To determine haematological counts, 50 μL of EDTA-anticoagulated PB were analysed using an Abacus Junior Vet Haematological Analyser (CVM, Navarra, Spain).

**Flow cytometry analyses**

Forty microlitres of PB samples or 5 × 10⁵ BM cells were stained with different moAbs, listed in Supplementary Table 1 (see Appendix), over 30 min at 4°C. After that, erythrocytes were lysed in ammonium chloride lysis solution (0.155 mmol/L NH₄Cl + 0.01 mmol/L KHCO₃ + 10⁻⁴ mmol/L EDTA). The stained cells were resuspended in flow cytometry buffer (PBS containing 0.5% BSA and 0.05% Azide) containing 1 μg/mL 4',6-diamidino-2-phenylindole (DAPI) as a viability marker and analysed in a BD LSRFortessa™ Cell Analyser (BD Biosciences, San Jose, CA, USA). A minimum of 10⁵ viable cells were acquired. Off-line analysis was performed using the FlowJo software package (TreeStar Inc, Ashland, OR, USA).

**LSK sorting**

HSCs with an LSK phenotype were purified from BM samples. The BM cells were lysed and stained for LSK phenotype using the moAbs listed in Supplementary Table 1 (see Appendix) and FACS-sorted using a BD Influx (BD Biosciences).

**Statistical analyses**

The statistical analysis was performed using GraphPad Prism Software version 5.00 (GraphPad Software, San Diego, CA, USA) for Windows. For all the analyses a non-parametric Mann–Whitney test was performed. Significances are expressed as *P* < 0.05(*)).

Power analyses was performed using DSS Research online tool (see https://www.dssresearch.com/KnowledgeCenter).

**Results**

To perform a direct comparison between the two selected routes for IV injection in the context of HSC transplantation we first performed a classical BMT in congenic mice. A scheme of the procedure can be observed in Figure 1a. vavΔhCD4-P3B mice (CD45.1⁺ΔhCD4⁺) were used as donors while P3B mice (CD45.1⁺ΔhCD4⁺) were used as recipients. A total of 3 × 10⁶ TBM cells were administered by either RO or TV injection into lethally-irradiated P3B recipients. Similar volumes were used for both injections in order that exactly the same conditions were achieved.

After transplantation, the recipient mice were analysed to assess donor PB engraftment. The analyses
revealed that, although almost full donor chimerism was achieved in all cases, RO-administered TBM cells provided significantly higher levels of engraftment than TV-administered cells both at 1 mpt (90.56 ± 2.42% vs. 85.32 ± 3.38%) and 3 mpt (92.4 ± 0.46% vs. 82.98 ± 11.78%) (Figure 1b). When BM was analysed at the endpoint of the experiment (3 mpt), these differences were not significant, as donor chimerism in both

Figure 1. Engraftment analyses in TBM transplants. A total of $3 \times 10^6$ vavΔhCD4-P3B TBM cells (CD45.1+ΔhCD4+) were transplanted into lethally-irradiated P3B mice (CD45.1+ΔhCD4+). Donor engraftment was determined at 1 and 3 mpt in PB, and at 3 mpt in BM when the animals were culled by the FACS analyses of the ΔhCD4+ population. (a) Scheme of the transplantation experiment. (b) PB donor engraftment. (c) Donor engraftment obtained in recipients’ BM at 3 mpt. (d) Donor LSK cell engraftment obtained in recipients’ BM at 3 mpt. (e) Lineage distribution of donor engraftment in recipients’ PB. (f) PB haematological counts at 3 mpt. Significance of differences between groups are expressed as $P<0.05$ (*). BM: bone marrow; FACS: flow activated cell sorting; LSK: Lin-, Sca1+, c-Kit+; mpt: months post transplant; n.s.: not (statistically) significant; PB: peripheral blood; PLT: platelets; RBC: red blood cells; RO: retro-orbital; TBM: total bone marrow; TV: tail vein; WBC: white blood cells.
TBM cells and LSK subpopulations was similar in both groups (Figures 1c and 1d). However, a more homogenous distribution of engraftment levels in the RO-injected group than in the TV-injected group was found, as shown by the coefficient of variation (0.50 vs. 1.40) (Figure 3a). Therefore, in a BMT study, similar PB and BM engraftment levels can be obtained with both RO and TV routes. These results were confirmed when haematopoietic reconstitution was studied. No significant differences were observed in the distribution of B cells, T cells or neutrophils between both groups (Figure 1e), or in the absolute numbers of leukocytes, erythrocytes and platelets (Figure 1f).

A power calculation analysis based on data from the analyses of the first month showed adequate power (0.94) with an alpha error level of 5% by using five mice in each group.

In BMT studies it is not always possible to treat recipients with a high conditioning regimen or to have a high number of cells available when using defined progenitors rather than TBM cells. To establish a different approach we decided to perform a BMT experiment in which conditions were not ideal so we could infer whether or not both administration routes were good under more restraining conditions. LSK cells from P3D2F1 mice (CD45.1+CD45.2+) were FACSPurified and transplanted in a limiting number (1500 cells per mouse) into sublethally-irradiated B6D2F1 mice by either RO or TV injection (Figure 2a). Similar volumes (200 μL) were used for both injections. After transplantation the recipient mice were analysed to assess PB engraftment using the CD45.1 and CD45.2 pan-leukocytic markers up to 3 mpt.

Donor chimerism level analyses revealed that LSK cells administered by RO and TV routes provided similar levels of engraftment at 1 and 3 mpt (RO engraftment levels: 24.53 ± 10.86% and 32.15 ± 10.82%, TV engraftment levels: 30.64 ± 14.82% and 37.44 ± 18.7%; Figure 2b). When BM was analysed at the end of the experiment, donor chimerism in both TBM cells and LSK subpopulations was also similar for both groups (Figures 2c and 2d). Although, again, a more homogeneous distribution of data was observed in the RO group (Figure 3b). These results were confirmed when haematopoietic reconstitution was studied in the recipients (Figure 2e). No significant differences were observed in either the distribution of B cells, T cells or neutrophils between both groups or in the absolute numbers of leukocytes, erythrocytes and platelets (Figure 2f).

Taking into account all these results, we can conclude that there are no significant differences between the engraftment levels obtained both in PB and BM with both IV injection routes. However, it is worth mentioning the higher homogeneity obtained in the results for the RO-injected mice in comparison with the TV-injected group, as shown by the values of the standard deviation and the coefficient of variation (Figure 3). In addition, no adverse effects were detected in any experiment.

Discussion

BMT is a procedure intended to replace damaged or ablated BM with healthy cells. This procedure may be used with different purposes in experimental mice: the study of different functions of haematopoietic and non-haematopoietic cells, studies of haematopoietic system regulation or ex vivo gene therapy protocols, among others.

A BM graft is delivered through the IV route. In mice the TV has usually been the most common choice. Other sites for delivery include the RO venous sinus, the BM cavity itself, and the spleen. The splenic route is not commonly used for delivering HSCs as it offers a very low rate of success in repopulating recipient BM in comparison with the other routes. The intra-bone delivery of HSCs is meant to increase the possibility of cells homing in the BM of the host, as they are delivered directly into the target tissue, thus avoiding homing through circulation. However, this is a very difficult procedure that requires expert manipulators, deep anaesthesia of the mice and a much reduced injection volume (up to 30 μL). Theoretically, one true HSC is sufficient for long-term BM engraftment. However, additional BM cells are required to ensure the survival of the mice in the early stages after transplantation as HSCs require time to engraft and differentiate into the different lineages. These support cells have to be co-administered, thus increasing the total volume necessary for inoculation. RO injection is easier to perform, but it requires the mouse to be anaesthetized and, as very little data are available regarding reconstitution after BMT, the route of choice is usually the TV.

In preparation for TV venipuncture, the mouse must be mechanically restrained and placed under a heat lamp to promote peripheral vasodilation. This approach can cause distress to the animals, especially if the initial venipuncture is unsuccessful and repeated attempts are made. If the needle is not correctly inserted inside the vein, cells will be delivered into the surrounding subcutaneous tissue, resulting in skin swelling. Also, in white mice the veins are generally easy to insert inside the vein, cells will be delivered into the surrounding subcutaneous tissue, resulting in skin swelling. Also, in white mice the veins are generally easy to detect as dark lines along either side of the tail, but in brown or black mice it is much more difficult to detect these lines. Of course, all of these drawbacks are minimized in the appropriate expert hands but they highlight the need for extensive training and expertise to
carry out this procedure with accuracy and reproducibility.

RO injection may seem aesthetically distasteful, but it is an easily-mastered technique and is ultimately more humane than the alternative IV injection technique in mice. It is easier and faster to perform as a mouse can be injected in as short as 15 s. Of course, it also has some drawbacks: the maximum volume injectable through this route can be a bit lower than through the TV, injection close to the eye-ball can be

Figure 2. Engraftment analyses in LSK cells transplants. A total of 1500 P3D2F1 LSK cells [CD45.1 + CD45.2 +] were transplanted into submyeloablated B6D2F1 [CD45.1 - CD45.2 +] recipients. Donor engraftment was determined at 1 and 3 mpt in PB and at 3 mpt in BM when the animals were culled by the FACS analyses of the CD45.1 + CD45.2 + population. [a] Scheme of the transplantation experiment. [b] PB donor engraftment. [c] Donor engraftment obtained in recipients’ BM at 3 mpt. [d] Donor LSK cell engraftment obtained in recipients’ BM at 3 mpt. [e] Lineage distribution of donor engraftment in recipients’ PB. [f] PB haematological counts at 3 mpt. BM: bone marrow; FACS: flow activated cell sorting; LSK: Lin-; Sca1+; c-Kit+; mpt: months post transplant; n.s.: not (statistically) significant; PB: peripheral blood; PLT: platelets; RBC: red blood cells; RO: retro-orbital; TV: tail vein; WBC: white blood cells.
disagreeable for some people, and there is the possibility that some cells or reagents might not be delivered in this situation.

In all these methods, manipulator skills are very important and can have great impact on the final results, even determining the difference between the success and failure of the graft. Thus choosing an easy procedure can lead to more reproducible and homogenous results.

Taking into account all of these considerations we have tried to perform a deep characterization of the RO injection-mediated BMT in mice. Two different approaches of syngeneic BMT were chosen: transplantation of high numbers of TBM cells and transplantation of limiting numbers of purified HSCs (defined as LSK cells). In both approaches we analysed engraftment levels, lineage distribution and haematological counts, trying to give an overall vision of the procedure.

We found that both RO and TV routes provided an equal haematopoietic reconstitution, as similar engraftment levels, lineage distribution and haematological counts were achieved. If anything, the RO route could provide higher levels of engraftment, as shown in the PB analyses of TBM-transplanted mice (Figure 1b). Remarkably, the level of variability among the different engraftment methods was lower in the RO-injected group (Figures 1c, 1d, 2c, and 2d). Both by the representation of PB engraftment in a box and whisker plot (Figures 3a and 3b) and by the calculation of the standard deviation value and the coefficient of variation (Figure 3c), we can clearly observe that the distribution of the results is much more homogeneous among the RO-injected mice than among the TV-injected ones, independent of the experiment type.

In conclusion, we have demonstrated that RO injection is a good alternative as an IV route for BMT in comparison with the more commonly used TV injection. It can reduce the time required for manipulation of the mice, thus reducing mouse and manipulator distress. The shortening of the procedure time can also be beneficial as cells will be in tubes for a shorter period prior to injections. As mentioned above, other studies have been published comparing the TV and RO administration routes that show similar results when delivering drugs, antibodies or cells in similar situations.2–6,9 Also, in our hands, RO administration has shown greater
consistency regarding donor chimerism levels due to the ease of the technique and the lower rate of failure.

Altogether, a higher homogeneity of data, equal or even higher engraftment values, and lower rates of failure offer significant improvement that could allow for a reduction in the number of animals used in a BMT experiment. Moreover, although both techniques cause mild suffering, RO injection is much easier to learn which in turn implies a reduction in the distress and suffering of the mice as it is performed under anesthesia. All of these considerations will eventually satisfy the principle of the 3Rs (replacement, reduction and refinement).

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Declaration of conflicting interests
The authors declare no conflict of interest.

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References


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### Appendix: Supplementary Table: List of antibodies used

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Hemorheology in experimental research: is it necessary to consider blood fluidity differences in the laboratory rat?

Ursula Windberger¹, Karl Spurny¹, Alexandra Graf² and Hansjörg Thomae³

Abstract
This study was designed to identify whether blood fluidity differs between commercially available laboratory rat strains. The hemorheological profiles of seven clinically healthy wild-type rat strains were analyzed to determine whether any diversity in blood fluidity might affect the outcome of cardiovascular studies. Study 1: 65 healthy adult rats (Lewis, Long–Evans, Hairless, Wistar and Fisher; mixed gender and comparable ages) were compared. In order to determine the greatest possible difference, the two strains with the greatest hematocrit (HCT) differences were selected for more detailed evaluation. Red blood cell (RBC) deformability (maximum elongation index, shear stress for half-maximal deformation of RBC; both \( P < 0.0001 \)), and the effect of plasma protein concentration upon plasma viscosity \( (P < 0.0001) \) were different between Lewis and Long–Evans strains. Whole blood viscosity – although different at native HCT \( (P < 0.004) \) – was unaltered following HCT standardization of samples. Differences in RBC aggregation were statistically significant but these were small and may not be clinically relevant. Study 2: these 65 animals were compared with 21 animals (10–16 weeks old; both sexes) from mutant strains (Dahl SS/JrHsdMcwiCrl, \( n = 10 \); ZDF-Leprfa/Crl, \( n = 11 \)). In both mutant strains, plasma and whole blood viscosity were increased compared with commonly used strains at native and standardized HCT \( (P < 0.001) \). Unusually high RBC aggregation values were seen in the ZDF rat strain \( (P < 0.001) \). It was concluded that the variability in blood fluidity among clinically healthy adult laboratory rat strains was both statistically and clinically significant. A hemorheological profile should be added to a routine phenotyping process, since both variables can significantly influence study outcomes.

Keywords
hemorheology, rat, phenotyping, RBC, cardiovascular

The fluidity of blood is based on the quantitative and qualitative properties of blood cells and the constitution of blood plasma. Compared with other particular systems or dispersions, the viscosity of whole blood is low, even in the presence of a 50% particulate fraction. This is due to the ability of red blood cells (RBCs) to contort according to hemodynamic forces. The cell membrane folds or aligns in the direction of flow, owing to the flexibility of the membrane cytoskeleton and the rotation of the phospholipid membrane around the cytoplasm. In addition to RBC size, the deformability of erythrocytes is also a crucial factor for microvascular perfusion. In association with the actual shear forces, blood cells aggregate or disaggregate when they pass through the vascular tree. Not every mammalian species, however, possesses RBCs with pronounced cell aggregability.¹,² If RBCs can form rouleaux, aggregation takes place at low shear rates

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(<10/s), whereas RBCs are singularly suspended in plasma at higher shear rates (>20/s).\(^3,4\) In vitro flow curves simulate this assembly and disassembly of RBCs. By contrast, there is also evidence for RBC aggregation under high shear forces, measured in vivo by ultrasound backscatter.\(^5\) RBC aggregation has been shown to affect the quantity of RBCs in subsequent vessels in the microvasculature.\(^6–8\) The modulation of endothelial shear stresses by the flowing blood has been proposed as an underlying causative mechanism. The cross-sectional shear rate gradient in a given vascular segment\(^9\) generates a phase separation of blood in the vessel – provided that RBC aggregation takes place. In this instance, the majority of RBCs would be located in the central layer, whereas the marginal layer would be cell-depleted. Using a numerical mode, a recent study has shown that both RBC aggregation and RBC deformability influence the organization of RBCs in the flow field in microvessels, resulting in phase separation.\(^10\) Such phase separation was also shown to be pronounced at low shear rates.\(^11\) Phase separation creates a marginal cell-free layer in the vessel lumen\(^12\) and the width of this cell-free layer affects the endothelial shear stress. As a result, vasoactive substances (nitric oxide, prostacyclin, endothelin) are expressed which adjust the arteriolar diameter.\(^13,14\) It should be considered, however, that cyclic blood flow instabilities may also happen through the pulsatility of blood flow and the arteriolar wall movement. The effect of RBC aggregation on blood flow has been summarized previously.\(^15\) This review shows that RBC aggregation has an impact on peripheral perfusion. It is therefore hypothesized that in the choice of animal for a specific experimental setting, differences in physiological RBC aggregability and deformability could bias the gained results.

The laboratory rat is an established species in experimental research and there are several strains available for different applications. The aim of the present study was to investigate the merits of using a particular strain in experimental applications with respect to hemorheology. Firstly, hemorheological values of arbitrarily chosen, frequently used wild-type rat strains were collected. These strains do not typically develop clinical illnesses during their adult lifespan. The data obtained from these animals were then compared with the values of two mutant wild-type rat strains (Dahl SS/JrHsdMew/Crl rat; ZDF-Leprfa/Crl rat). All animals were clinically healthy at the time of blood collection. The results from this study showed differences in blood fluidity among wild-type rat strains, which are relevant to experimental applications.

### Animals

A total number of 86 adult rats were included in the study.

Five strains (65 animals) of the wild-type genome were subsequently identified in the text as ‘commonly used’ strains. Both sexes were used and the animals were 10–16 weeks old (see Table 1 for the demographic data). All animals were kept conventionally in polyethylene cages mounted by filter tops in the animal house of the Medical University of Vienna. The Lewis rat strain was housed in a specific pathogen free (SPF) enclosure. The animals were kept on a 12 h day/12 h night cycle starting at 06:00 h and 18:00 h, respectively. All the animals except the Lewis strain had been inbred over a long time period throughout the animal house. These strains had been bred for maintenance and training purposes for the education of animal keepers. By contrast, the Lewis rats were purchased from Charles River Inc (Sulzfeld, Germany) at intervals of 2–3 years (which equals 8–12 generations) in order to maintain the LEW/Crl phenotype. For this reason, F3–F12 generations were used for this study. The animals were fed conventional rat diets (treated by autoclave: V1324–300, or extruded: V1326–000; ssniff, Soest, Germany) and tap water ad libitum. The health status of the animals was recorded on a regular basis (the last complete parasitology, bacteriology and virology tests had been undertaken three months prior to the experiment). The Lewis rats inside the SPF barrier followed the Federation of European Laboratory Animal Science Associations.
(FELASA) SPF recommendations and were free of parasites, viruses, and bacteria. The rats, which were kept conventionally in filter top cages, were free of ectoparasites and worms, but they were positive for protozoa (Spironucleus sp.). These rats were negative for viruses specified on the FELASA list, but colonized by Pasteurella pneumotropica, Staphylococcus sciuri, and Corynebacterium bovis. All the rats were examined by the veterinarian prior to the experiment and were considered clinically healthy. They showed no clinical signs of infection.

Two mutant strains (21 animals) were used: Dahl SS/JrHsdMcwiCrl (n = 10, 5 females + 5 males) and ZDF-Leprfa/Crl (n = 11, 6 females + 5 males). The animals were 10 weeks old when they arrived in the animal house and were kept conventionally in polysulfone cages mounted by filter tops. The mutant strains were fed the same diet (V1326–000; ssniff) and tap water ad libitum. These animals were tested within the first four weeks of arrival in the animal house; with the exception of one female ZDF rat, which was tested at 16 weeks of age.

The rats were kept in the animal facilities of the Medical University of Vienna in accordance with institutional policies and federal guidelines.

Materials and methods

Experimental procedure

The rats were bled by cardiac puncture, under general anesthesia with 100 mg/kg ketamine and 10 mg/kg xylazine intraperitoneally. Blood (5–10 mL) was withdrawn smoothly from each animal using a 10 mL syringe mounted by a 16 G needle. Blood samples were collected into tubes containing sodium EDTA, sodium heparin, and sodium citrate. Animals were killed by cervical dislocation following the sampling procedure. In order to eliminate circadian influences on blood fluidity, blood samples were withdrawn in the morning between 08:00 and 10:00 h.16 The procedure was approved by the Animal Experimental Ethics Committee of the Medical University of Vienna and the Austrian Federal Ministry of Science and Research (animal license number: GZ-66.009/0158-II/3b/2013).

Hematology and blood chemistry

A routine hematological profile was obtained from each animal using the Cell-Dyn 3500 (Abbott, Abbott Park, IL, USA). Hematological parameters included mean corpuscular volume (MCV, in fL), mean corpuscular hemoglobin (MCH, in pg/mL), mean corpuscular hemoglobin concentration (MCHC, in %) and red and white blood cell count (RCC, in cells·10¹²/L, WCC, in cells·10⁹/L). Hematocrit (HCT, in %) was measured by centrifugation (Hettich, Tuttingen, Germany).

The routine blood chemistry profile of each animal (Hitachi 904; Hitachi, Tokyo, Japan) was performed to show the health status of the animals. Plasma fibrinogen concentration (FIBR, in mg/dL) was measured manually according to the Clauss method.

Hemorheology

Whole blood viscosity (WBV, in mPa·s) was carried out using the LS30 viscometer (Contraves AG, Zürich, Switzerland) at 37°C. Blood samples were firstly measured at native HCT values, then centrifuged for HCT (40 ± 2%) adjustment with autologous plasma as described1 and analyzed again.

Principle of measurement: A one millilitre sample of blood was placed into the modified Couette cylinder system of the device. The viscosity of blood exerts a mechanical strain on the free movable cylinder, which was measured electromagnetically. Shear rates of 0.7, 2.4 and 94/s were used in this study. Three consecutive readings were recorded for each value.

RBC aggregability was measured using the Myrenne aggregometer MA1 (Myrenne, Roetgen, Germany) at room temperature and native HCT (aggregation index: M0, M1). Principle of measurement: A 30 μL sample of blood was placed between the transparent cone-plate shearing device and spread to a cellular layer. This layer was first sheared at 600/s for 10 s to disaggregate all pre-existing aggregates, then abruptly stopped in M0 mode or rotated at 3/s in M1 mode for another 10 s to allow aggregation. The extent of plasma gaps inside the whole blood monolayer allowed light transmission through the red cell suspension. The instrument computes an aggregation index (M0 at stasis and M1 at 3/s) proportional to the area under the light transmission curve.17 At least five readings were taken for each value of M0 and M1.

Erythrocyte deformability was measured by laser diffraction analysis, using an ektacytometer (LORCA; Mechatronics, Hoorn, The Netherlands). For various fluid shear stresses, elongation indices (EI) were calculated from the diffraction image as the (length – width)/ (length + width) of the image at 37°C. The shear stress for half-maximal deformation (SS½) and the EI at the highest shear stress (EI max) were estimated as described elsewhere.18

Principle of measurement: RBC deformability was measured at fluid shear stresses between 0.3 and 53.06 Pa. Twenty-five microlitres of blood was diluted in a phosphate buffered saline (pH 7.4) – polyvinylpyrrolidone (PVP, M = 360,000 Sigma-Aldrich, Germany)
medium of known viscosity (25.7 mPa-s). The sample was sheared in the Couette system with a 0.3 mm gap between the two cylinders. A laser beam traverses the suspension and gets diffracted by the RBCs in that area. EI was calculated for each shear stress.

Plasma viscosity (PV, in mPa-s) was measured by OCR-D (Anton Paar, Graz, Austria) at 25°C. Principle of measurement: one milliliter of plasma was filled into a plastic cup and mounted at the bottom of a glass capillary (diameter: 0.9864 mm, length: 100 mm). The plastic cup was compressed at 2 Hz exerting shear rates inside the capillary. The viscosity at the shear rate of 10/s was read for PV.

**Experimental design and statistics**

In the first study, the two commonly used rat strains with the greatest deviation in HCT were selected for further exploration. This was done in order to define the greatest possible differences in hemorheological values between laboratory rats of comparable age. The sample size calculation for the number of rats was based on pilot data for the Long–Evans (lowest HCT) and Lewis (highest HCT) groups of the variable WBV0.7s-1 available from three rats per group. The pilot data showed a mean of 34.4 mPa-s in the Lewis group and 27.5 mPa-s in the Long–Evans group with a common standard deviation of 7.2. Using a two-sided t-test (with significance level 0.05), a sample size of 20 per group was found necessary to detect the given difference with 80% power. The 20 animals per group were drawn randomly from the given pool. Unpaired t-tests were used to investigate the differences in hemorheological parameters (WBV0.7s-1, WBV2.4s-1, WBV_94s-1, PV, M0, M1, EI_{max}, SS_{½}), MCV and total protein (TP). To correct for multiplicity, all P values less than 0.05 were considered as statistically significant (Bonferroni adjustment). In addition, a similar analysis was calculated to investigate sex-related differences among the commonly used rat strains. To investigate the correlation between TP, PV and WBV0.7s-1, regression analysis for TP accounting for rat-group, PV, and WBV0.7s-1 was performed. A similar analysis was calculated to investigate the coherence between M1 and fibrinogen. Pearson correlation coefficients were calculated. The other strains were investigated by descriptive statistics only.

In the second study, comparisons between commonly used and mutant strains were made. All data collected in Study 1 (65 animals) were pooled and compared with the ZDF group (11 animals) and the Dahl/SS group (10 animals) separately by analysis of variance (ANOVA). The following parameters were tested: WBV0.7s-1, WBV2.4s-1, WBV_94s-1, PV, M0, M1, EI_{max}, SS_{½}, TP and MCV. To correct for multiplicity, again, all P values less than 0.005 were considered statistically significant. Again, as an addition, t-tests were used to compare parameters between male and female rats. Furthermore, a two-way ANOVA with factors rat strain and sex was performed. To correct for multiplicity, all P values less than 0.005 (i.e., 0.05/10) were considered as statistically significant.

In both studies, descriptive statistics were calculated using the median, 25% and 75% percentiles. Statistical analysis was performed using SAS (release 9.1; SAS Institute Inc, Cary NC, USA).

**Results**

Figure 1 shows the RBC EI–shear stress curves of Lewis and Long–Evans rats. Figure 2a and b shows the aggregation indices M0 and M1, Figure 3 shows the PV and Figure 4a and b show the WBV of the rat strains.

Table 2 shows the hemorheological data, HCT, MCV, TP, cholesterol (CHOL) and fibrinogen (FIBR) of those rat strains that were included into the statistical evaluation (Lewis, Long–Evans, ZDF and Dahl/SS rat strains). The complete data-set of all commonly used rats is provided in the supplementary file (supplementary material is available online with this article, http://lan.sagepub.com).

**Hemorheological differences in the commonly used rat strains [Study 1]**

The greatest differences in HCT were observed between the Lewis and Long–Evans rats. These two strains were therefore selected for the statistical analysis. HCT was chosen as a key parameter as it is a major determinant of blood viscosity. By comparing native blood at different HCT values, WBV2.4s-1 was found to differ (P = 0.004) between the two rat strains. This observation necessitated the standardization of all samples to a specific HCT to diminish the effect of HCT and RCC on blood fluidity. Therefore, only HCT standardized blood samples were included into the analysis (WBV at native HCT is shown in the supplementary file, Table B). At the standardized HCT values, no significant differences were observed in WBV between Lewis and Long–Evans rat strains. No significant differences in PV were observed between these strains. TP was found to have a significant influence on values of PV (P < 0.0001; Pearson correlation coefficient: 0.80) for these two rat groups. No similar influence on the values of WBV0.7s-1 could be detected. Aggregation index M0 (P = 0.005) was increased in the Lewis rat strain, however, the aggregation indices M1 did not differ significantly. RBC deformability parameters (SS_{½} and EI_{max}, both P < 0.0001) were increased in
the Lewis rat strain. Since geometric factors (for instance, cell size) influence the EI, as measured by ectacytometry, it is important to control MCV. For rat strains in this study, no significant differences ($P = 0.17$) in MCV were seen, so the influence of cell size on RBC deformation may be disregarded. The ‘maximum’ RBC deformation ($E_{I_{\text{max}}}$) was higher in Lewis rats, although the cells required a higher shear stress to reach half of this ‘maximum’ deformation. Figure 1 shows that the ‘maximum’ deformation did not reach a plateau value; so the highest EI in this chart does not provide a real ‘maximum’ deformation of the RBC. This effect has also been observed previously for the elephant.19 In our method, the shear stress range that was presented in the sample was fixed. However, extrapolation of the EI–shear stress curve towards higher shear stresses resulted in a plateau at higher EI values in the Lewis rat. By considering both indices together ($SS_{1/2}$ and $E_{I_{\text{max}}}$) we suggest that a higher shear stress is needed for initial RBC deformation in Lewis rats. When those RBCs have undergone 50% of their deformation, their further elongation is alleviated compared with that of Long–Evans rats.

Hemorheological differences between the commonly used and the mutant rat strains [Study 2]

In this study, the hemorheological values of Dahl/SS and ZDF rats were compared with the pooled values of all other rat strains. WBV was increased in the ZDF rat strain at all shear rates ($P < 0.0001$). In the Dahl/SS rat strain, WBV was elevated at the lowest shear rate (0.7/s) only ($P < 0.001$). PV was increased in both mutant strains (both $P < 0.0001$). Aggregation indices M0 and M1 were also increased (all $P < 0.001$). $SS_{1/2}$ and $E_{I_{\text{max}}}$ of the mutant strains did not differ significantly from those of commonly used strains. MCV, however, was lower in the ZDF rat strain ($P < 0.001$). A smaller cell requires a higher shear stress for the same deformation than a larger cell if the membrane has the same flexibility. Therefore, the lack of response does not implicate that RBC deformability in the ZDF rat strain was unaltered compared with commonly used rat strains.

In addition, the effect of sex on the variables has been tested. There was a sex-related difference in $WBV_{94s^{-1}}$ in the Lewis rat ($P < 0.0001$). Significant larger parameters were found for female rats. There were no differences in the Long–Evans rats. However, $WBV_{94s^{-1}}$ did not show a significant difference between the Lewis and Long–Evans rats in our main study. A two-way ANOVA with factors rat strain and sex confirmed no significant differences between female and male rats within the rat strains ZDF, Dahl/SS, and the pooled values of all other rat strains. Medians and interquartile distances of variables, separated by sex are provided in Table C of the supplementary file.

Other laboratory values

Commonly used strains. Differences in blood chemistry were observed between strains. The plasma CHOL (mg/dL) concentration, for example, was higher in the Lewis rats than in the Long–Evans rats. Although the aggregation index M0 differed between the two strains, FIBR (mg/dL) was similar. Hematology was similar.
between the two strains, with the exception of RCC. In principle, hematology of the commonly used strains was within the range of our laboratory specific reference values for the ‘rat’ species. These were also comparable with reference values from the literature.\textsuperscript{20,21} Deviations from reference values (laboratory reference values, and Ref. 22) concerning the plasma concentration of liver transaminases and specific ions were observed in the Hairless rat strain (see Table A in the supplementary file). These differences existed despite the animals having the same diet as the other strains of this study and their being kept in the conventional part of the animal house.

**Mutant strains.** The plasma concentrations of CHOL and FIBR were higher than in the commonly used strains. TP was increased in the ZDF rat strain ($P < 0.0001$) only. All other values were comparable with the reference values of the vendor.

**Discussion**
Cardiovascular physiology and fluid dynamics investigations may require precise blood fluidity values. The relevant parameters for obtaining blood fluidity in the experimental animal of choice should therefore be known. The laboratory rat is frequently used

**Figure 2.** Aggregation indices M0 (a) and M1 (b) of the rat strains.
in biomedical research. Several different rat strains are available from academic animal houses and commercial suppliers. The selection of the optimal rat model is often done habitually or is based on cost or availability. Whether differences in blood fluidity exist between laboratory rat strains has not been previously evaluated in a coherent study. Most importantly, whether such differences can significantly influence the outcomes of studies with cardiovascular or fluid mechanics components has not yet been established.

The phenotype of rats generally differs across breeds. If kept in an isolated animal house for a period of years, the characteristics of a rat strain from a specific source will change over time. If a strain is reproduced in different facilities over an extended time span, hemorheological parameters may vary for that reason. Changing the breeding modalities from outbred to the easier workable inbred type may further modify the animal’s phenotype. The first aim of this study was to investigate whether any differences in hemorheological parameters are present within commonly used wild-type laboratory rat strains (Study 1). This question is best addressed when the phenotypes of the strains are as diverse as possible. Rat strains which had been isolated for many generations in our animal facility were therefore included. The (originally outbred) Long–Evans strain, for example, has been inbred in our animal house for more than 30 years. These strains had been kept conventionally and although their phenotypical features had been maintained, diversifications in biochemistry between strains may have occurred over that time. Hematology and blood chemistry are provided in the supplementary file. Animals purchased from a commercial vendor at regular intervals were also included in order to avoid a change of phenotype during reproduction in our animal house. These animals were kept in an SPF enclosure.

As HCT is the major determinant of blood viscosity, the two strains identified as having the maximal physiological HCT difference were selected for Study 1. The ‘in-house’ Long–Evans rat strain was found to have the lowest HCT among the five available strains and the Lewis rat strain to have the highest. The hemorheological parameters of these two strains were compared in order to determine whether differences exist between healthy rat strains.

RBC deformability and aggregability were found to differ between these two strains. The differences in RBC aggregability were small and may not be clinically relevant. The differences in RBC deformability, however, may have some clinical importance. Lewis rat strain RBCs required a greater shear stress for their initial deformation than those of the Long–Evans rat strain. Once they reached half of their ‘maximum’ deformation, however, they showed a greater extended symmetry. This diversity was unexpected. RBC transit time in capillaries is affected by cell deformability in addition to cell size. As such, it is recommended that RBC deformability be tested in studies where the microvascular blood flow is analyzed in rats. RBC deformability may also affect RBC alignment in the flow field and in the formation of the marginal cell-free layer in blood vessels. The cell-free layer width was shown to be associated with the endothelial nitric oxide synthase

![Figure 3. Plasma viscosity (PV) of the rat strains.](image)
(eNOS) expression via the endothelial shear stress stimulus\textsuperscript{12} and the distribution of RBC in subsequent vessels.\textsuperscript{6}

The differences in RBC deformability and aggregability were found to have no influence on apparent WBV, as indicated by the flow curves: HCT standardized blood samples were compared. Similar WBV values were observed in standardized HCT samples for the two healthy strains. If differences in the flow curves of healthy rat strains are obtained, the possibility should therefore be considered of this finding being due only to the effect of the HCT.

Plasma TP concentration is the major determinant of PV. Variations of TP during an experiment using rats would therefore modify the animal’s PV to different degrees. Variability in the influence of TP on the values of PV was found in Lewis versus Long–Evans rat strains. This could be important for the expression of endothelial vasoactive factors, given that PV contributes significantly to endothelial shear stress.\textsuperscript{23}

In Study 2 it was hypothesized that the hemorheological parameters of rat strains which typically develop specific diseases within a time span after birth, may be different from those that have a normal life expectancy. This is hypothesized to be the case even when animals are clinically healthy at the time of examination. For this reason the results of Study 1 (all 65 rats) were pooled together and compared with the values of two

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{Whole blood viscosity [WBV\textsubscript{0.7}\textsuperscript{s\textsuperscript{-1}} (a) and WBV\textsubscript{94}\textsuperscript{s\textsuperscript{-1}} (b)] of the rat strains.}
\end{figure}
Table 2. Hemorheological values at standardized hematocrit (HCT) (40%, centrifuged cells in autologous plasma).

<table>
<thead>
<tr>
<th></th>
<th>Lewis</th>
<th>Long–Evans</th>
<th>ZDF</th>
<th>Dahl/SS</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBV0.75s−1 (mPa·s)</td>
<td>30.81 (23.45/35.93)</td>
<td>27.95 (21.86/29.92)</td>
<td>42.30 (37.05/48.68)§</td>
<td>39.63 (25.51/60.78)§</td>
</tr>
<tr>
<td>WBV2.4s−1 (mPa·s)</td>
<td>18.49 (15.12/20.83)</td>
<td>17.62 (15.26/20.56)</td>
<td>23.46 (19.54/25.89)§</td>
<td>21.49 (16.22/29.23)</td>
</tr>
<tr>
<td>WBV94s−1 (mPa·s)</td>
<td>5.84 (5.18/6.17)</td>
<td>5.55 (5.11/6.39)</td>
<td>7.56 (7.06/8.13)§</td>
<td>6.30 (5.74/6.74)</td>
</tr>
<tr>
<td>PV (mPa·s)</td>
<td>1.22 (1.19/1.24)</td>
<td>1.23 (1.19/1.26)</td>
<td>1.48 (1.39/1.56)§</td>
<td>1.31 (1.28/1.53)§</td>
</tr>
<tr>
<td>M0</td>
<td>0.9 (0.5/2.2)*</td>
<td>0.3 (0.1/0.6)*</td>
<td>3 (1.5/5)§</td>
<td>1.5 (0.5/5)§</td>
</tr>
<tr>
<td>M1</td>
<td>5.6 (3.5/8.2)</td>
<td>4 (2/8)</td>
<td>13 (9.5/15)§</td>
<td>8 (4/19)§</td>
</tr>
<tr>
<td>EImax</td>
<td>0.67 (0.66/0.69)*</td>
<td>0.59 (0.57/0.59)*</td>
<td>0.64 (0.63/0.64)</td>
<td>0.63 (0.63/0.64)</td>
</tr>
<tr>
<td>SS½</td>
<td>2.84 (2.54/3.39)*</td>
<td>1.71 (1.56/2.15)*</td>
<td>2.85 (2.61/3.35)</td>
<td>2.07 (1.86/2.20)</td>
</tr>
<tr>
<td>HCT (%)</td>
<td>45 (42/48)</td>
<td>37 (34/39)</td>
<td>41 (38/48)</td>
<td>41.5 (41/44)</td>
</tr>
<tr>
<td>TP (g/dL)</td>
<td>5.9 (5.5/6.4)</td>
<td>5.7 (5.5/6.0)</td>
<td>7.7 (6.8/8.5)§</td>
<td>5.9 (5.6/6.6)</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>55.8 (54/56.8)</td>
<td>56.8 (54.3/58.8)</td>
<td>52.3 (51.7/53.5)§</td>
<td>57.4 (55.8/61.1)</td>
</tr>
<tr>
<td>CHOL (mg/dL)</td>
<td>82 (77/94)</td>
<td>61.5 (54/65)</td>
<td>135 (114/151)</td>
<td>91.5 (80/149)</td>
</tr>
<tr>
<td>FIBR (mg/dL)</td>
<td>184 (169/211)</td>
<td>226 (174/260)</td>
<td>276 (236/340)</td>
<td>286 (242/342)</td>
</tr>
</tbody>
</table>

The table also shows the native HCT, as well as total protein (TP), mean corpuscular volume (MCV), cholesterol (CHOL), and fibrinogen (FIBR) of blood samples of the tested rat strains. Values are expressed as median and 25%/75% percentile in parenthesis. WBV: whole blood viscosity, PV: plasma viscosity, M0, M1: aggregation indices, EImax: elongation index at the highest shear stress, SS½: shear stress for half-maximal deformation. *Values different (P < 0.005) between commonly used rat strains (Lewis versus Long–Evans); Study 1. §Values of mutant rat strains different (P < 0.005) from the pooled data of all other rats; Study 2.

Values such as these may occasionally be observed in aged male OFA rats weighing more than 700 g (personal observation). Since the HCT was standardized, the high WBV values in the flow curves of the ZDF rats are likely to be the result of this augmented RBC aggregability, at least at the low shear rates.

The high RBC aggregation of the mutant rats was the most noteworthy finding in Study 2. RBC aggregation is associated with blood flow. Blood flow velocity and the vessel radius determine the vascular shear rate. This in turn regulates rouleau formation. RBC aggregability itself was postulated to be associated with the width of the marginal cell-free layer in the flowing blood. A low viscosity of the fluid layers that are nearest to the endothelium cells would thereby reduce the endothelial wall stress. When a reduced endothelial wall stress is transmitted to the endothelial cell, in the presence of a regular endothelial cell phenotype, this leads to a modulation of the vessel diameter. The cell-free layer width—which shows spatial variations at bifurcations—is also shown to influence the dissipation of RBC into subsequent vessels. This effect may occur through plasma skimming of daughter vessels. Findings from a number of authors support this hypothesis: (i) RBC aggregation was found to modulate the intramyocardial HCT gradient in guinea pig hearts; (ii) RBC aggregation was found to influence the flow resistance in isolated striated muscles; (iii) RBC aggregability was shown to affect the vaso-motor tone via eNOS expression; (iv) the blood flow in a given vascular segment was found to affect the HCT in the subsequent vessels; and (v) the preferred perfusion of the faster branch at arteriolar bifurcations was found to affect the tissue HCT in striated muscle of obese Zucker rats. Based on these findings it is proposed that the dissipation of RBC in the peripheral vasculature will be altered by the enhanced RBC aggregation in our mutant rat strains. It is proposed that this effect will be pronounced when the animals develop clinical signs of disease. It is further hypothesized that both RBC ‘feeding’ into subsequent vessels and nitric oxide production in endothelial cells may then be reduced.

Sex-related, as well as gender-related, differences in laboratory values are an important issue in laboratory animal research. This study was not designed to investigate sex differences among the hemorheological variables.

mutant strains, the ZDF rat and the Dahl/SS rat. Both of these mutant strains were provided by the vendor. The mutant strain populations included 11–16-week-old animals of both sexes, which were not yet showing clinical signs of their disease. The plasma concentrations of TP, FIBR and CHOL in these mutant rat strains were already enhanced, although the animals were clinically unremarkable. These parameters are known to influence RBC aggregation and membrane deformation. Significant increases in WBV, PV and RBC aggregation were observed in the ZDF and Dahl/SS strains relative to the commonly used strains. The aggregation index M1 in the ZDF rat strain reached unusually high values for the rat species. Values such as these may occasionally be observed in aged male OFA rats weighing more than 700 g (personal observation). Since the HCT was standardized, the high WBV values in the flow curves of the ZDF rats are likely to be the result of this augmented RBC aggregability, at least at the low shear rates.

The high RBC aggregation of the mutant rats was the most noteworthy finding in Study 2. RBC aggregation is associated with blood flow. Blood flow velocity and the vessel radius determine the vascular shear rate. This in turn regulates rouleau formation. RBC aggregability itself was postulated to be associated with the width of the marginal cell-free layer in the flowing blood. A low viscosity of the fluid layers that are nearest to the endothelium cells would thereby reduce the endothelial wall stress. When a reduced endothelial wall stress is transmitted to the endothelial cell, in the presence of a regular endothelial cell phenotype, this leads to a modulation of the vessel diameter. The cell-free layer width—which shows spatial variations at bifurcations—is also shown to influence the dissipation of RBC into subsequent vessels. This effect may occur through plasma skimming of daughter vessels. Findings from a number of authors support this hypothesis: (i) RBC aggregation was found to modulate the intramyocardial HCT gradient in guinea pig hearts; (ii) RBC aggregation was found to influence the flow resistance in isolated striated muscles; (iii) RBC aggregability was shown to affect the vaso-motor tone via eNOS expression; (iv) the blood flow in a given vascular segment was found to affect the HCT in the subsequent vessels; and (v) the preferred perfusion of the faster branch at arteriolar bifurcations was found to affect the tissue HCT in striated muscle of obese Zucker rats. Based on these findings it is proposed that the dissipation of RBC in the peripheral vasculature will be altered by the enhanced RBC aggregation in our mutant rat strains. It is proposed that this effect will be pronounced when the animals develop clinical signs of disease. It is further hypothesized that both RBC ‘feeding’ into subsequent vessels and nitric oxide production in endothelial cells may then be reduced.

Sex-related, as well as gender-related, differences in laboratory values are an important issue in laboratory animal research. This study was not designed to investigate sex differences among the hemorheological variables.
in the rat strains, and indeed the sample size may be too low to detect significant differences. It is important to note that the results of this study are not modified if the factor of ‘sex’ is included in the analysis. No significant hemorheological differences between sexes in the mutant and commonly used rat strains were observed. Recent studies have demonstrated that sex has an influence on hemorheological parameters in an outbred rat strain as well as in humans. The results from mutant rats in the current study support these previous findings as they provide a typical tendency, although they lack consistency. The influence of sex and gender on the hemorheological values of laboratory animals is an important issue for future investigation.

In conclusion, differences in blood fluidity were observed among the different laboratory rat strains. RBC membrane flexibility can be diverse in healthy wild-type animals, which are commonly used in biomedical research. This should therefore be measured if the phenotype of the strain is going to be described. The varying degree to which plasma protein influences PV may be of interest for the experimental surgeon/analyst or during clinical applications. In rat strains, which develop a disease or abnormal pathology after a certain period, the hemorheological variables may differ profoundly from those of other wild-type strains. This is the case even if they are clinically healthy at the time of examination. Generic values cannot be applied to these rats; and each strain needs to be tested separately. It is possible that this effect may be pronounced in genetically-modified strains. The results from this study show that RBC deformability and RBC aggregability may significantly influence outcomes in cardiovascular studies. It is therefore recommended that these parameters should be measured during an animal’s phenotyping.

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The authors declare that there is no conflict of interest.

References


Coil optimization for low-field MRI: a dedicated process for small animal preclinical studies

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Abstract
We demonstrate a method for the fast in vivo quantification of small volumes, down to 25 μL, using low-field magnetic resonance imaging (MRI) coils. The coils were designed so as to maximize the signal-to-noise ratio (SNR) in the images. For this we developed an analytical model for describing the variations of the SNR with coil design and with size/shape suited to the object under observation. Based on the conclusions drawn from the model, the coil parameters were chosen in order to reach an SNR close to the maximum. For the validation of the model, coils were finally characterized in terms of quality factor using saline phantoms. The coil design procedure is illustrated here with two examples: first, the quantification of about 200 μL of intradermal injected gel on rabbits with a single loop surface coil and second, the imaging of the intervertebral disks in rat tails using a small volume coil to detect possible lesions. Such studies would not have been feasible for the clinical low-field MRI system at our disposal using any of the commercially available medium-sized manufactured coils. As a result of this simple optimization procedure, a wide range of applications is accessible even at low magnetic fields, leading to new opportunities for low-cost, though efficient, preclinical studies.

Keywords
low-field MRI, coil optimization, non-invasive, small animal, sacrifices reduction

Preclinical studies usually result in the sacrifice of a high number of animals. But preclinical imaging, especially preclinical magnetic resonance imaging (MRI), offers an attractive non-invasive alternative. Animals can be followed in vivo in longitudinal studies, thus allowing the number of animals needed to be reduced.¹ For most studies, small animals such as rodents or lagomorphs are used. But preclinical imaging using a clinical whole body MRI using the commercial coils that come with the system is not always optimal. This is especially the case if none of the coils amongst the set of coils provided by the manufacturer of the MRI device properly matches the animal shape. Moreover, third party companies do not always offer commercial dedicated coils for small or medium-sized animal imaging in clinical MR systems. Examinations of small animals are often performed over small regions of interest (ROI), and only a weak signal is available with a non-dedicated coil, especially at low magnetic field strengths (below 0.5 Tesla [T]). Even if preclinical imaging focused on small animals employs dedicated high magnetic field MRI (4.7 T, 7 T or higher), these MR systems are expensive and are not always accessible in institutions.² Moreover, the overall gain in image quality is not as high as expected.³,⁴ Image quality at clinical low fields (e.g. 0.2 T or 0.4 T) has been proven to be suitable for small animal studies when optimized coils were employed.¹,⁴ The advantages of clinical low-field systems are that the open architecture offers the possibility of performing interventional surgery. They also lead to a reduction in set-up times and are less hazardous with regard to magnetostatic forces. Moreover, no...
specific absorption rate (SAR) issues, which cause tissue heating, are reported, and less susceptibility artifacts are observed. This study offers an alternative to the preclinical imaging of rodents currently performed on high-field preclinical or clinical systems, by employing an inexpensive 0.18 T open magnet designed for human extremities. The cost of this system is three to six times lower than that of clinical wide bore systems (1.5 T and 3 T). Besides the low initial cost, the maintenance cost for a low-field permanent magnet is almost insignificant, notably because of the absence of power consumption and cryogenic fluids in the magnet. This clinical low-field system is also most suited for animals such as lagomorphs, due to its open magnet and its 200 mm × 200 mm × 200 mm static field and radiofrequency (RF) excitation field homogeneous region. In this work, two specific in vivo preclinical applications were assessed, and a dedicated coil was designed for each application. First a circular-shaped loop surface coil was built to quantify intradermic volumes of gels (∼200 µL) injected into rabbit dermis. Intradermal follow-up is usually performed by histology measurements which require the euthanasia of animals at regular time intervals during the study. Although the use of ultrasound imaging has been reported, MR is a non-invasive alternative for tracking the volume of intradermal injections. A similar study has been notably reported using an expensive 1.5 T clinical MRI device. Secondly, a volume coil was developed to image the tail of the rat in order to observe intervertebral disks and to further detect potential lesions. The degeneration of the caudal intervertebral disks is frequently monitored using MR. But this kind of study is usually performed using clinical or preclinical high-field MRI devices (>3 T). Low-field MR was therefore envisaged for this study as well as for the lagomorph study.

Materials and methods

Animals

The preclinical study on a rabbit model was requested by a third party institution for the commercialization of a hyaluronic gel designed for plastic surgery. The experimental protocol complied with all animal welfare guidelines, and with the regulations on animal experimentation (Directive 86/609/EC, amended by decree 87-848, decrees 2001-464 and 2005-264) and the recommendations of the Council of Europe Convention EST123. Five New Zealand White adult female rabbits (Charles River Laboratories International, Inc, Wilmington, MA, USA) with a mean weight of 3.5–4 kg were housed in the Claude Bourgelat Institute (VetAgro Sup, Lyon, France). Each of the five rabbits was injected in the dermis over eight sites with four different hyaluronic acid gels, one control reference gel and three gels under test. MR (followed by image processing for volume calculation) of all 40 intradermal injection sites was performed on day 0 (d0, injection), day 28 (d28) and day 84 (d84). The rabbits were anaesthetized during the MRI examinations using 1% isoflurane pushed by air with a cone-shaped head holder. The rabbits lay in a left decubitus position and their body temperature was maintained with hot water bottles.

The caudal disks were imaged in vivo on a Wistar Unilever adult rat. The animal lay in a prone position during the MR acquisitions and was anaesthetized with a 1% isoflurane/air mix with a well-adjusted cone-shaped head holder. Its body temperature was maintained with a hot water bottle on contact with the end of the tail.

Instruments

An MRI E-scan XQ device (Esaote, Genova, Italy) with an open permanent magnet and vertical static magnetic field of magnitude $|B_0| = B_0 = 0.18$ T ($f_0 = 7.78$ MHz) designed for human extremities was used for both studies. The transmit-only coil was a saddle coil embedded into the device. Gradient coils which were available were able to reach amplitudes up to 20 mT/m, which made high spatial resolution imaging accessible (higher than 1 pixel/mm in each direction). Receive coils at our disposal were separate from the transmit coil on this MRI device and were decoupled from each other with a passive and/or a geometric method. The dedicated coils which were designed in the present paper employed either two axial-led 1N5819 Schottky or two surface-mounted DH80055 PIN crossed diodes (Temex Ceramics, Pessac, France) for decoupling. Decoupling of the receive coil during RF pulses from the transmit coil was needed to avoid image distortions.

Coil optimization

With the commercial coils available in the clinical low-field MR system, small ROI could not be explored in acquisition times compatible with in vivo examinations. This was due to the small level of signal available in a reduced ROI, especially in low-field MRI. Two studies were consequently carried out: first on a surface coil, because for some applications it is crucial to obtain as much signal as possible over a reduced ROI at the skin surface, rather than to get an image of the whole body with an average signal-to-noise ratio (SNR); secondly on a volume coil for which the shape of the coil embraces the volume of the sample. For both coils,
the design process was divided into three steps: mathematical modeling for the optimization of coil performance, coil fabrication, and finally electrical characterization based on electronic parameters to check performance and validate the model.

**Mathematical model.** The SNR intrinsic to an MRI coil was first described by Hoult\(^15\) as in (Eq. 1):

\[
SNR \propto \frac{|B_{1r}|}{\sqrt{R_{eq}}}
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where \(B_{1r}\) (T/A) is the current sensitivity of the coil (dependent mainly on coil geometry) and \(R_{eq}\) (Ω) is an equivalent resistance in series with the inductance which summarizes overall loss phenomena occurring in the coil (e.g. a larger coil has a higher \(R_{eq}\)).\(^16\) \(B_{1r}\) is the field induced by a unit current flowing in the coil by reciprocity.\(^16\) At low fields and thus long wavelengths, a quasistatic approach (the Biot–Savart law) remains accurate for estimating \(B_{1r}\).\(^16,17\) Integration using the Biot–Savart law was performed numerically using MATLAB (The MathWorks, Inc, Natick, MA, USA) over volumes or surfaces specified by the constraints of the study. During the MRI experiment, the amplitude of the signal collected by the receive coil originating from a specific location was maximized when \(B_{1r}\) at the center of the coil was orthogonal to \(B_0\) at this location. If \(B_0\) is arbitrarily oriented such that \(B_0 = B_0y\) and is considered as perfectly homogeneous in both strength and direction, only the \(x\) and \(z\) components of \(B_{1r}\), \(B_{1x}\) and \(B_{1z}\) are considered. In (Eq. 1), \(|B_{1r}|\) becomes \(B_{1xz}\).\(^18\)

\[
B_{1xz} = \sqrt{B_{1x}^2 + B_{1z}^2}
\]

For some applications, for example in volume coils, an image with the most constant signal over a given region is needed. As a consequence, it is useful to evaluate the maximum deviation of the collected MR signal in this region thanks to the principle of reciprocity.\(^15\) Assuming that the sample is homogeneous and that the RF field produced by the transmit-only coil is also homogeneous over this volume, the spatial variations of the collected MR signal are caused only by the spatial variations of sensitivity in the receive coil. We therefore introduce the maximum relative deviation \(\Delta\) of \(B_{1xz}\) as:\(^18\)

\[
\Delta = \max \left(\frac{B_{1xz}(r_0) - B_{1xz}(r_1)}{B_{1xz}(r_0)}\right)
\]

with \(r_0\) as a reference point and \(r_1\) a point in the field of view (FOV) of the coil which maximizes the deviation. For volume coils, \(\Delta\) is calculated on the \(z\) axis.\(^18\) Thus, \(r_0\) refers to the center of the coil where \(B_{1xz}\) is at a maximum, and \(r_1\) is the coil boundary on the axis where \(B_{1xz}\) is at a minimum. This definition was extrapolated to surface coils: at a given surface distance \(z_0\), the maximum deviation of \(B_{1xz}\) in a plane parallel to the coil is easily computed. This plane is bounded to the projected edges of the coil. With this definition of \(\Delta\) for surface coils, \(r_0\) and \(r_1\) respectively refer to the points of maximum and minimum for \(B_{1xz}\) at depth \(z_0\).

For the estimation of \(R_{eq}\), the schematic of Figure 1 includes the various elements involved. This equivalent circuit was needed for the estimation of \(R_{eq}\) and subsequently for the calculation of the SNR variation (Eq. 1).

The overall noise sources can be described by various contributions\(^19\) (see Appendix A): the losses in the coil itself (\(R_{coil}\)), the losses in the sample under observation (\(R_{sample}\) which is subdivided into two distinct phenomena: the magnetic losses \(R_m\) and the dielectric losses \(R_d\)) and finally the losses in the electronic components (such as the tuning capacitors \(R_t\) and decoupling diodes \(R_{dec}\)).\(^16,20–22\) The losses in the coupling capacitors can be ignored if the coil is tuned and matched at \(f_0\).\(^20\) The losses in the coaxial cable can be ignored as well. These two assumptions were confirmed by circuit simulations in LTspice (Linear Technology, Milpitas, CA, USA) and ANSYS HFSS (ANSYS Inc, Canonsburg, PA, USA). At \(f_0 = 7.78\) MHz, the wavelength is about 39 m in air and 1.35 m in muscle,\(^23\) which were much greater than coil or sample dimensions in our studies. The radiation losses were consequently considered negligible. For the same reason, the equivalent electronic circuit of Figure 1 was valid. Apart from these considerations, every loss phenomenon needs to be taken into account at this working frequency.\(^16,24–26\) Thus, coil and sample losses for medium-sized (1–10 cm diameter) coils are of the same order of magnitude, by contrast with the high-field experiments where sample losses or even tuning capacitor losses can become significant.\(^16,21,26\) At \(B_0 = 0.18\) T, a substantial increase in SNR is therefore achievable in theory by reducing coil losses, i.e. by optimizing receive coil design. In order to identify and avoid the main contributions of SNR degradation in our MR experiments, an exhaustive modeling of the losses as well as of the coil sensitivity map were necessary (Eq. 1).\(^15,20\)

For a given FOV, constrained by the sequence parameters and hardware characteristics, and for any given object or ROI which needs to be examined with its specific shape and size, the geometry of the coil is usually chosen either for filling factor (and thus SNR) maximization or for minimization of \(\Delta\). Once the geometry has been determined, a compromise between \(R_{eq}\) reduction and \(B_{1xz}\) increase (Eq. 1) can be achieved.
Figure 1. Electronic circuit of a low-field magnetic resonance imaging (MRI) device (coupling capacitors not shown). (a) All the individual loss phenomena are illustrated: \( R_{\text{coil}} \) and \( R_{\text{leads}} \) are the losses in the coil itself, \( R_m \) and \( R_d \) are losses caused respectively by the magnetic and dielectric losses in the sample, and \( R_{\text{dec}} \) and \( R_{\text{ct}} \) are the losses in the electronic components (respectively the decoupling diodes and the tuning capacitor). (b) The equivalent parallel RLC circuit. (c) The hybrid equivalent circuit which includes the equivalent series resistance \( R_{eq} \) required for the estimation of the signal-to-noise ratio (SNR). For circuit (c), the series inductance is almost identical to that in parallel, provided that the quality factor \( Q_0 \) of the coil \( (R_{eq}/\omega_0 + L_{eqP}) \) is much larger than unity. Then \( R_{eq} = R_{eqP}(1 + Q_0^2) \) with \( Q_0 = \omega_0 L_{eq}/R_{eq} \). The equations used for the calculation of all parameters are reported in Appendix A.
by adjusting the remaining free geometrical parameters of the coil (such as the number of turns in a solenoid or conductor thickness). This compromise is not always straightforward, since increased sensitivity can be obtained at the expense of increased losses. Simulations using a simple though exhaustive SNR model are therefore valuable for the optimization of the design of a coil dedicated to a specific application.

Concerning the study on rabbit dermis, a dedicated receive surface coil was designed so as to image and quantify the intradermal gel volumes. Single circular loop geometry was initially chosen for this application because of the shape of the sample whose projection on the dermis and coil plane ($x_0z$, see Figure 2) was circular, but egg-shaped on an orthogonal plane ($x_0y$ for example). The circular coil was compared to a square coil with a side equal to the diameter of the circular coil. The values of SNR and $\Delta$ were estimated for both surface coils. The SNR was estimated on the axis $z_0$ of the coil, at depth $z_0$ which stood for the maximum extent of the injection into the dermis of the rabbit. The calculation of $B_{1xz}$ maximum relative
deviation $\Delta$, using Eq. 3, was computed along a line parallel to the $x$ axis at $z = z_0$ (see Figure 2a). The radius $a$ of the loop was first constrained by the ROI which was a 15 mm wide injection. Consequently, the coil radius was at least 8 mm. Secondly, the maximum depth $z_0$ of about 10 mm also determined the loop radius for the best examination affordable in the dermis. Indeed, the SNR at depth $z = z_0$ was maximized by choosing the best $a$ value. The SNR variation for a simple circular geometry was well described by Suits et al.\textsuperscript{16} in 1998. They considered the case of a semi-infinite medium with negligible dielectric sample losses. However, for a complete understanding of the various noise contributions, SNR was expressed here using Eq. 1 and Appendix A. The coil sensitivity $B_{1\text{sz}}$ of the circular-shaped coil was calculated over the $x0z$ plane with $-20\text{ mm} < x < 20\text{ mm}$ and $0\text{ mm} < z < 25\text{ mm}$. Figure 2 illustrates the results of the simulations both for the spatial (Figure 2a) and parametric variations of $B_{1\text{sz}}$ (Figure 2b).

The SNR of a circular surface coil strongly depends on the radius as expected.\textsuperscript{14} As observed in Figure 2b, SNR($a$) for a given depth $z_0$ shows a maximum for a unique $a_{\text{opt}}$ value. The dots on each curve of Figure 2b stand for the maxima of SNR($a$) for various observation depths $z_0$. For $z_0 = 10\text{ mm}$, we found that $a_{\text{opt}}$ was $\sim 10\text{ mm}$. The $B_{1\text{sz}}$ map (Figure 2a) confirms that for such dimensions, the coil includes in its FOV the whole injection site (dashed ellipse). According to complementary simulations at depth $z_0$ on the axis of a square coil with a side of $20\text{ mm}$ the SNR was $9\%$ higher, whereas $\Delta$ was $25\%$ lower compared to a circular coil with a radius of $10\text{ mm}$. Yet, the SNR on the axis at $z = 0$ was $19\%$ better for the circular coil. Although a circular geometry was chosen initially, these simulations suggest that for this application, a square-shaped coil would be a good alternative offering slightly better performances. The wire was chosen as the thickest available in the laboratory for $R_0$ minimization, i.e. $dw = 1.5\text{ mm}$. This diameter is the maximum for enameled copper wire available at most electronic retailers. Although 2 mm is also available, the goal of this paper was to allow for the in-house design of low-field RF coils using off-the-shelf materials, so this copper wire was employed.

Concerning the study on caudal intervertebral disks, MR of the rat tail was performed in order to observe disk quality and identify potential lesions for preclinical studies in which caudal disk regeneration in rats would be followed.\textsuperscript{12} According to the shape and size of the tail, the most appropriate coil geometry was a cylindrical volume coil. With vertical low static field $B_0$, the coil type allowing for the best intrinsic SNR and lowest $\Delta$ is of a solenoid type.\textsuperscript{1,5,29,30} As a compromise between $\Delta$ and SNR performances needed to be reached, variations of both relative to the design parameters of the coil were undertaken. The four parameters for a solenoid-shaped coil are the length $L$ of the solenoid, the radius $R$ of the solenoid (distance from conductor center to solenoid axis), the wire thickness $dw$, and the number of turns $n$. On the one hand, the SNR model was derived from Eq. 1, for which $R_{\text{eq}}$ was estimated as in Appendix A. On the other hand, $\Delta$ was calculated between the points at $r_0 = \{0, 0, 0\}$ (the center of the solenoid) and $r_1 = \{0, 0, L/2\}$ (the edge of the solenoid)\textsuperscript{18} (Figure 4a). The parametric variations of $\Delta$ and SNR are illustrated in Figure 3. Each was obtained as a function of one of the four parameters of the coil, the remaining three being set to arbitrary values (those used in the final design of the coil).

According to the model (Figures 3c and 3d), $\Delta$ depends only on the length $L$ and the radius $R$ of the solenoid, and these two parameters were set first. The radius $R$ of the coil was fixed at 11.75 mm, given the diameter of the tail was up to 15 mm, which allowed a 1.5 mm air margin on both sides for ease of insertion and took into consideration the thickness of the coil’s plastic holder (namely 2 mm on both sides, which gives an inner diameter of 18 mm). Two non-successive intervertebral disks were injured by a needle in previous studies,\textsuperscript{10,12} consequently a length $L$, allowing an FOV over a ROI with five vertebrae, was found to be practical for observing two non-successive disks simultaneously. A good compromise between $\Delta$ and SNR performance was achieved if the length of the solenoid $L$ was approximately equal to the length of the ROI (Figure 3a). We finally set $L$ at 45 mm (Figure 4a). At this point, two free parameters remained: $n$ and $dw$. The SNR variation versus $n$ and $dw$ in Figure 4b indicated that the best SNRs would be achieved with thick wire ($dw$ between 2 mm and 5 mm) and a limited number of turns ($n$ between 7 and 11) for an SNR within 10% of the optimum.

With the $dw = 1.5\text{ mm}$ wire, only $n$ could finally be adjusted. The best value of $n$ for these three constrains was $n = 10$, as is illustrated in Figure 3d and Figure 4b. For this design, the map of the spatial variations of $B_{1\text{sz}}$ is presented in Figure 4a.

**Coil fabrication.** For the surface coil, the conclusions of the model led to the design of a circular-shaped coil with $a = 10\text{ mm}$ and $dw = 1.5\text{ mm}$ for the best SNR at a depth $z_0 = 10\text{ mm}$. A picture of the completed surface coil is shown in Figure 5a.

Figure 5b illustrates the constructed volume coil. The regularity of space between two consecutive turns was achieved by manually winding a plastic wire along with the copper conductor wire before applying glue. Once the glue was dry, the plastic wire was removed.
For the electronic part of the surface and volume coils, the tuned impedances (without the matching capacitors) were respectively $8.9 + j * 128 / C_{10}$ and $6.6 + j * 965 / C_{10}$. Tuning at $f_0 = 7.78 \text{ MHz}$ (with $C_t$) and matching at $Z_0 = 50 \Omega$ (with $C_m$) – the transmission line characteristic impedance – were performed by ATC (American Technical Ceramics, Huntington Station, NY, USA) case ‘100A’, ‘700A’ or ‘100B’ (for high capacitance components) non-magnetic ceramic/porcelain capacitors (Figure 5 and Appendix A). A symmetric pattern was chosen for the matching network in order to reduce dielectric sample losses, especially for the volume coil. Signal transmission was provided by a one-meter long RG58 coaxial line with non-magnetic BNC connectors (Radiall, Aubervilliers, France). To avoid excitation field inhomogeneity, the experiments with the small surface coil were set up in order to achieve a geometrical decoupling in addition to the passive decoupling diode, because the signal induced in the loop during transmit pulses was too weak. This coil orientation was manageable due to the open architecture of the MRI magnet and the vertical $B_0$ orientation. However the size of the volume coil was sufficient for the induction of a sufficiently high voltage across the decoupling diodes during transmit pulses.

A +36 dB low noise (1.3 dB) preamplifier AU-1466 from Miteq (Miteq Inc, Hauppauge, NY, USA) was inserted between the coil and the MRI device to increase the signal of interest, thus minimizing parasitic noise. The amplifier was kept outside $B_0$ for proper working, namely around 500 mm away from the center of the magnet.

**Electrical characterization.** When tuned and matched on a vector network analyzer (VNA) E5071 model from Agilent (Agilent Technologies, Santa Clara, CA, USA), quality factor measurements were performed to characterize the coils at room temperature ($22^\circ \text{C}$) either when unloaded ($Q_{\text{meas},U}$) or loaded ($Q_{\text{meas},L}$) with a phantom. Quality factors $Q_{\text{meas},U/L}$ were measured with the VNA on scattering parameter $S11(f)$ curves over the Smith chart using a dedicated MATLAB script. This program was able to accurately estimate $Q_{\text{meas}}$ and the coupling coefficient $\kappa$ between the coil and the VNA as a result of an effective procedure. When the coil was either unloaded (index ‘U’) or loaded (index ‘L’) with the

![Figure 3. Normalized signal-to-noise ratio (SNR) and maximum relative deviation $\Delta$ of $B_{1xz}$ for a solenoid when: (a) length $L$ varies; (b) radius $R$ varies; (c) wire thickness $dw$ varies; (d) number of turns $n$ varies. The SNR was normalized against the final design which is $L = 45 \text{ mm}$, $R = 11.75 \text{ mm}$, $dw = 1.5 \text{ mm}$ and $n = 10$. The constants are: the radius $Rs$, the length $Ls$ and the dielectric properties of the sample under observation.](image-url)
phantom, the actual quality factors of the disconnected coil (unplugged from the VNA), $Q_{0,\text{meas},U}$ and $Q_{0,\text{meas},L}$, could be deduced from the measured values $Q_{\text{meas},U}$ and $Q_{\text{meas},L}$ through:

$$Q_{0,\text{meas},U} = \left(1 + \frac{1}{C_{20}}\right) / C_1$$

$$Q_{0,\text{meas},L} = \left(1 + \frac{1}{C_{20}}\right) / C_1$$

If impedance matching is lossless and perfectly adjusted to $Z_0$, the coupling coefficient $\kappa$ is 1, and we confirmed the well-known fact that $Q_{0,\text{meas}}$ is twice $Q_{\text{meas}}$. In this particular case, $Q_{0,\text{meas}}$ is twice the ratio between the peak frequency $f_0$ and its bandwidth $\Delta f$ at $-3\text{ dB}$.\textsuperscript{3,30} Phantom loading was obtained with a 0.45% NaCl solution phantom adapted to the coil type (a 10 mL tube for the volume coil, and a 170 mm $\times$ 170 mm $\times$ 170 mm parallelepiped filled with an $\sim$4.9 L solution for the surface coil). This phantom has electrical properties which simulate living tissues, especially muscle properties (relative permittivity $\varepsilon' \sim 208$, conductivity $\sigma \sim 0.61 \text{ S/m}$).\textsuperscript{23}

For a comparison between the measurements and the SNR model, $Q_{0,\text{calc},U/L}$ values were calculated according to the estimation of the electrical components depicted in Figures 1c and 1b as:

$$Q_{0,\text{calc},U} = \frac{\omega_0 L_{eq}}{R_{eq,U}} \quad \text{and} \quad Q_{0,\text{calc},L} = \frac{\omega_0 L_{eq}}{R_{eq,L}}$$

Or:

$$Q_{0,\text{calc},U} = \frac{R_{eq,U}}{\omega_0 L_{eq,U}} \quad \text{and} \quad Q_{0,\text{calc},L} = \frac{R_{eq,L}}{\omega_0 L_{eq,L}}$$

MRI

A validation study was performed in vitro prior to the experiments on the rabbits in order to assess the accuracy and repeatability of the volume quantification using MRI. This validation study consisted of the MR acquisition of a microtube filled with a 100 $\mu$L volume of water using a 200 $\mu$L micropipette, followed by volume quantification. The volume measurement procedure (including acquisition and quantification) was repeated nine times by the same operator for an estimation of the repeatability while the accuracy was...
calculated as the difference between the measured and expected volumes. The surface coil was then compared to a commercial volume coil from the device manufacturer (a quadrature receive-only knee coil) both in vitro (on a saline phantom) and in vivo (on an injected rabbit) in terms of SNR. The SNR was measured as the mean value of the signal in the ROI divided by the standard deviation of the signal in the background of the image.34 The coil was set close to each injection site of each rabbit (Figure 6a).

For SNR characterization, both the commercial knee volume coil (Figure 7e) and the designed surface coil (Figures 6b and 7d) were employed for the MRI of an injection site. The acquisition sequence was a 3D gradient echo sequence with TR = 26 ms, TE = 10 ms, NEX = 2, FA = 85°, matrix = 256 × 256 × 52/FOV = 130 mm × 130 mm × 31.2 mm. The coil was set close to each injection site of each rabbit (Figure 6a).

The quantification of the volumes of the sites on all the rabbits was performed using ImageJ software (Rasband WS, US National Institutes of Health, Bethesda, MD, USA) on threshold-based segmented images (Figure 6c). The segmentation was assisted by manual corrections which consisted in the delimitation on each slice of a coarse area in which the threshold-based segmentation was then carried out. Volume calculation and 3D rendering were finally obtained using a MATLAB script (Figure 6d). Volume variation between d0 and d28, then between d0 and d84, were estimated as (V(d28) − V(d0))/V(d0) and (V(d84) − V(d0))/V(d0), respectively. Given the small statistical sample considered, a Wilcoxon signed-rank test was carried out for the follow-up of each gel volume between d0 and d28, and d28 and d84, and then between d0 and d84.

For MR imaging on the rat, the tail was inserted into the volume coil to image the first caudal vertebrae. The acquisition sequence was a 3D gradient echo sequence with TR = 50 ms, TE = 16 ms, NEX = 1, FA = 20°, matrix = 192 × 192 × 52, FOV = 120 mm × 120 mm × 31.2 mm (voxel volume of 0.625 × 0.625 × 0.6 mm³), and for an acquisition time of 8 min 19 s. For this sequence, the estimated readout time was about 30 ms, giving a 30–40 Hz/pixel receive bandwidth. This sequence was optimized through flip angle choice to get the best contrast for intervertebral disks. SNR comparison was performed between this volume coil and the surface coil designed for the rabbit study in which the tail was inserted.

Results and discussion
In preclinical imaging on small and medium-sized animals such as rats or lagomorphs, there is no low-field MRI device (B₀ below 0.5 T), apart from 1.0 T and 1.5 T devices that are costly and are usually considered to be low-field preclinical MRI devices. Clinical whole body systems, which are not always available for research and preclinical purposes, are even more costly. The use of a low-field (0.18 T) clinical MRI device dedicated to veterinary and preclinical practice with an open magnet sufficiently wide for lagomorphs (~200 mm) offered an alternative to conventional preclinical MRI and with several advantages. First the initial and maintenance costs of the device are reduced (averaging around €0.35M for a clinical 0.2 T,
compared with about €1M for a clinical 1.5 T and nearly €2M for a clinical 3.0 T). The use of a clinical device dedicated to animals has the advantage of avoiding the issues related to hygiene or allergies if regular clinical MRI devices are used with animals. The main advantage of low-field MRI is that, since the sample losses are reduced compared to those of high-field experiments (>0.5 T), the optimization of coils can generate a large increase in SNR. The use of optimized coils with medium-sized or small animals permits the generation of high spatial resolution images within an acceptable acquisition time. Low-field MRI is therefore well adapted for many preclinical studies. Moreover, the high cost of commercially supplied coils may encourage the design and conception of home-made dedicated coils. However the limitations of low-field systems are the low SNR which results if receive coils are not optimized, and an inability to perform studies such as spectroscopy.

For both studies performed on the clinical 0.18 T MRI device, the measured and calculated values of the actual quality factors $Q_0$ for the surface and volume coils are reported in Table 1.

![Figure 7. Signal-to-noise ratio (SNR) comparisons performed on: (a), (b), (c) a saline phantom and (d), (e), (f) in vivo on rabbit dermis. The SNR was measured along a 30 mm long line orthogonal to the surface (dashed lines on the magnetic resonance images). The measurements with the dedicated surface coil are represented by the images in (a) and (d) and are plotted in (c) and (f) as the continuous upper curve. The measurements with the volume knee coil were performed on images (b) and (e) and are presented as the lower dashed curves in (c) and (f). The location of the circular-shaped coil is displayed by the small circles right above the surface standing for the cross-section of the loop in (a) and (d).](image)

**Table 1.** Measured and calculated values of the actual quality factor $Q_0$ for the surface and volume coils when unloaded ($Q_{0,U}$) and loaded ($Q_{0,L}$) with a 0.45% saline solution.

<table>
<thead>
<tr>
<th></th>
<th>$Q_{0,U}$</th>
<th></th>
<th>$Q_{0,L}$</th>
<th></th>
<th>$Q_{0,U}/Q_{0,L}$</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Measured</td>
<td>Calculated</td>
<td>Measured</td>
<td>Calculated</td>
<td>Measured</td>
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<tr>
<td>Surface</td>
<td>98 ± 1</td>
<td>102 ± 1</td>
<td>98 ± 1</td>
<td>98 ± 1</td>
<td>~1.00</td>
</tr>
<tr>
<td>Volume</td>
<td>199 ± 1</td>
<td>198 ± 1</td>
<td>199 ± 1</td>
<td>191 ± 1</td>
<td>~1.00</td>
</tr>
</tbody>
</table>
Measured or calculated $Q_{0,U}/Q_{0,L}$ ratios were close to unity for the two coils. In other words, sample losses are negligible compared to coil losses. This was to be expected, given the small size of the coils\textsuperscript{15,16}. The advantage of such an outcome is that the coils remain matched to $Z_0$ when samples with electrical characteristics close to biological tissue are inserted. The comparison between measured and calculated $Q_0$ factors for both coils showed a discrepancy of less than 5% both when they were and were not loaded with a phantom, which validated the model. According to these simulations, the parasitic losses in the two coils did not account for more than 7 mΩ in series with the loop. Yet, this would represent 47% of the overall losses in the surface coil (see Appendix A, Figure 10a). Accurate simulation of such small coils is therefore crucial as the SNR is dependent on small loss phenomena. The quality factors of both coils would have been slightly increased using a thicker copper wire, or using wire with a rectangular cross-section. An analytical model for this kind of wire in a loop coil was notably proposed by Li et al.\textsuperscript{35} However wires with a rectangular section are usually more difficult to purchase.

In the MRI experiments on rabbits for the intradermal gel study, a comparison between the designed surface coil (10 mm radius) and the commercial knee coil (~100 mm radius) is presented in Figures 7a to 7c and 7d to 7f, respectively.

**Figure 8.** In vivo volume measurements at day 0 (d0, blue), day 28 (d28, red) and day 84 (d84, green) of the four gels injected in the rabbit dermis (n = 5 rabbits). A significant swelling ($P < 0.1$) was measured from d0 to d28, and also from d0 to d84. See online version for all colour references.

**Figure 9.** Rat tail images for the in vivo examination of intervertebral structures with (a) the volume coil and (c) the single turn surface coil both in sagittal view. As a result of 3D acquisition, the same images were also observable in transverse view when cutting on the dashed line, as illustrated in (b) and (d) for volume and surface coil, respectively (ImageJ Volume Viewer). The transverse view is obtained without significant loss in image resolution as a result of the quasi-isotropic acquisition. The scale is identical for all four images.
At point \( \{x, y, z\} = \{0, 0, z_0\} \), with \( z_0 = 2 \text{ mm} \) the distance from surface coil center to skin (or phantom) surface, the ratio between the SNR of the surface coil and the mean SNR inside the commercial knee volume coil was around 14 both on the phantoms (Figure 7c) and in vivo (Figure 7f). For the volume quantification, it was essential that the volume of the voxels was minimized (0.51 × 0.51 × 0.6 mm\(^3\) was the minimum accessible with the gradients of the MRI device employed here) to reduce volume quantification error. It was also important that the total acquisition time remained short to minimize anesthetic hazard and improve rabbit recovery. A scan time of 12 min 41 s for each injection site was considered a good compromise to allow the acquisition of images with a sufficient SNR (~80) using the surface coil. Based on these considerations, both with regard to spatial resolution and SNR, the study would not have been feasible using the commercial coils at our disposal (mean SNR ~ 8, see Figure 7c), or it would have required a scan duration 100 times longer.

The in vitro validation study gave for the 100 \( \mu \text{L} \) tubes a repeatability of 4.7% and an accuracy of 0.5%. The MR acquisition protocol associated with the dedicated surface coil was therefore appropriate for the rabbit study with volumes ranging from 130 \( \mu \text{L} \) to 420 \( \mu \text{L} \).

The results of the in vivo volume measurements are summarized in Figure 8. Significant increases (\( P \) value <0.1) in the volumes of the four gels were measured from d0 to d28, as well as from d0 to d84. However, no conclusions could be drawn, according to the statistical tests, concerning differences in volume increases between any of the four gels.

MR images performed on microtubes (for the validation study), on phantoms (for comparison with the commercial coil), and on rabbits showed that the SNR provided by the surface coil with the 3D sequence was suitable for quantifying the volume of injected gels, making it possible to follow volume variations in the gels. Yet, some injection sites had issues – for example some of them leaked or failed, some injections were subcutaneous instead of intradermal, and some even leaked outside the body. The volume of these sites (20 out of 40) was therefore not measurable. Statistical results over the 20 measurable volumes showed that all four gels swelled by 85% with a standard deviation of 24% between d0 (initial volume 200 \( \mu \text{L} \)) and d84. This high standard deviation was due to the small number of measurable volumes (5 per gel) and the locations of the injection sites which varied from one animal to another, probably inducing variability in the response of the bodies. The intradermal injections also changed in shape or size across the study, which was to be expected. Nevertheless, the ROI was still included in the FOV of the coil, as presented in Figure 6d, and the volume quantification was still possible. When designing the surface coil, a radius security margin could have been an option (by increasing the radius by 20%, for example), but that would be at the expense of a decrease in coil sensitivity. For this study, we chose to maximize the SNR at a depth of 10 mm into the rabbit and reduce the radius of the coil at its minimum, i.e. 10 mm.

Concerning the volume coil dedicated to the rat tail, the model confirmed that for solenoid-shaped coils of this size, coil losses are still dominant. Reducing the radius \( R \) to its minimum for this specific rat breed would ultimately maximize SNR, but the available margin (1.5 mm on both sides for the largest tails) was found to be practical for the experimental set-up. Sequence parameters were chosen to provide a good SNR (\( = 126 \pm 1 \)) on intervertebral disks (Figures 9a and 9b) along with a good spatial resolution (0.625 mm/pixel in-plane and 0.6 mm of slice thickness). In comparison, the SNR obtained with the rat tail in the surface coil was 109 ± 1 over the same ROI (Figures 9c and 9d).

Given the definition of a volume coil, the latter was preferred to the single loop surface coil since \( B_1^* \) variations are significantly smaller, which made it more suitable for this application. Moreover, it was particularly valuable to easily localize a specific disk and to be able to observe five of them at a time. The intervertebral disks of these rats was measured at approximately 25 ± 2 \( \mu \text{L} \) (the voxel volume being about 0.23 \( \mu \text{L} \)), which was in accordance with literature.

**Conclusion**

Clinical low-field MRI has great potential, especially for those longitudinal preclinical studies which otherwise usually require a large number of animals to be euthanized over time. Indeed, clinical MRI allows the use of a reduced number of animals in non-invasive longitudinal studies of medium-sized and small animal models. In this work, analytical SNR models for coil designs have revealed the importance of small coil optimization for low-field MRI, since sample losses become negligible. Using these easily built home-made coils at 0.18 T, the images in both studies described here had an SNR (>100) suitable for the accurate measurement of small volumes, with a satisfactory image acquisition time of about 10 min. Accurate quantification of small volumes varying from 200 \( \mu \text{L} \) down to 25 \( \mu \text{L} \) is thus possible with low-field MRI. The design procedure proposed in this paper is clearly applicable to other low-field strengths, such as at 0.4 T, where the SNR is intrinsically better.
Acknowledgements

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References

Appendix A: Loss contributions in a low-field MRI coil

The expressions of the various loss resistances (Figure 1) related to the various dissipative effects that impair the overall SNR (see Eq. 1) are presented below for both the surface coil and the volume coil.

Circular-shaped coil

For the circular loop coil (Figure 5a), seven loss contributions were considered. First the losses in coil conductor are 

$$R_{coil} = \rho \frac{2\pi R}{A}$$

(6)

with $R$ the radius of the coil, $A$ the area of the cross-section of the coil’s conductor in which the RF current effectively flows, accounting for the skin effect$^{15,20}$ and $\rho$ the resistivity of copper at ambient temperature ($1.74 \times 10^{-8}$ S/m at 23°C). The losses in the leads (i.e. the copper strips from the coil conductor to the tuning capacitor on the printed circuit board [PCB]) are $R_{leads}$:

$$R_{leads} = \rho \frac{L}{A_L}$$

(7)

with $l_L$ = the length of the two leads, and $A_L$ the area of the cross-section of the copper strips in which the RF current effectively flows, also accounting for the skin effect. Concerning the sample, the inductive losses $R_m$ are:

$$R_m = \frac{1}{2} \sigma \cdot \frac{\mu_0^2}{A_0^2} \cdot R_3$$

(8)

with $\mu_0$ the vacuum permeability, $\sigma$ the sample conductivity, and $A_0$ the working pulsation (i.e. $2\pi f_0$). $R_m$ was estimated through:

$$R_m = \sigma \int_{\text{sample}} |E|^2 \, dV$$

(9)

The dielectric losses in the sample $R_d$ were neglected for this coil geometry. The losses in the tuning capacitor, however, were estimated. The equivalent series resistance $R_C$, of tuning capacitor $C_t$ was fitted by:

$$R_C = a \cdot f_0^b \cdot C_t^c$$

(10)

with $\{a$, $b$, $c\}$ the model parameters, fitted from ATC capacitor datasheets. For example, the ATC100A case leads to $\{a$; $b$; $c\} = \{3.83 \times 10^{-10}$; $0.5$; $-0.3762\}$ (fit goodness $R^2 = 0.9973$). The RF losses in the two antiparallel passive decoupling diodes with zero biasing $R_{dec}$ were calculated. $R_{dec}$ was deduced from quality factor measurements on the coil, with and without the decoupling diodes. We obtained for the Schottky diodes employed for this surface coil $R_{dec} = 0.49 \Omega$ with $C_{dec} = 300$ pF. Based on the result of the fitting process, the parasitic losses $R_{parasit}$ were estimated at a value of about 7.6 m\(\Omega\). Figure 10a summarizes the relative loss contributions in the circular loop coil, calculated as the power dissipated in the various equivalent resistors. For this coil, we calculated the inductance of the loop $L_{eqP} = 31 \mu H$ and $L_{eqP} \sim 150 \Omega$ (see Figure 1). Thus, the final capacitance values were 13.27 nF for the tuning capacitor $C_t$, and 585 pF for each coupling capacitor $C_m$.

Solenoid-shaped coil

For the solenoid-shaped coil (Figure 5b), seven loss contributions were also considered. The losses in the coil conductor were estimated similarly to the surface coil as:

$$R_{coil} = \phi \cdot \rho \cdot \frac{(2\pi R + L)}{A}$$

(11)

with $n$ the number of turns, $L$ the length of the solenoid, $R$ the radius of the solenoid (from the axis of the coil to
with $L_S$ the length of the sample, and $R_S$ the radius of the sample.\textsuperscript{16,20} In our study, we arbitrarily constrained $L_S$ to $2L$ and $R_S$ to a constant value of 5 mm. The dielectric losses in the sample $R_d$ were calculated with:\textsuperscript{20}

$$R_d = \frac{\varepsilon'}{\alpha_0 C_2 \varepsilon''}$$

(13)

with $\varepsilon = \varepsilon' + i \varepsilon''$ the complex permittivity of the dielectric (i.e. the sample). It should be noted that $\varepsilon'' = \sigma / \alpha_0$. $C_1$ and $C_2$ in Figure 1 are calculated as a proportion of the overall stray capacitance $C_{\text{stray}}$ of the solenoid-shaped inductor:

$$C_1 = \frac{1}{2} \frac{C_{\text{stray}}}{1 - f_d} \quad C_2 = \frac{1}{2} \frac{\varepsilon' C_{\text{stray}}}{f_d}$$

(14)

with $f_d$ being the dielectric filling factor, which was linearly fitted from the data collected by Minard and Wind\textsuperscript{20} and Van Heteren et al.\textsuperscript{38} The stray capacitance is given by:\textsuperscript{37}

$$C_{\text{stray}} = 10^{-10} K_1 \cdot 2R$$

(15)

with:

$$K_1 = 0.08 + 0.1126 \frac{L}{2R} + \frac{0.27}{\sqrt{L/2R}}$$

(16)

Others have suggested models for the estimation of $C_{\text{stray}}$, which gave in our case rather similar values in the range of 1 pF.\textsuperscript{27,39} Concerning the losses in the tuning capacitor, $R_{\text{ct}}$ was calculated as in Eq. 10 for the circular-shaped loop coil. The RF losses in the two anti-parallel decoupling diodes with zero biasing $R_{\text{dec}}$ were measured similarly to the circular loop coil. We found that $R_{\text{dec}} = 1362 \Omega$ in series with $C_{\text{dec}} = 2.9 \text{pF}$. The parasitic losses $R_{\text{parasit}}$ were estimated to 10 mΩ. Figure 10b illustrates the relative loss contributions in the solenoid-shaped coil.

For this coil, an inductance $L_{\text{eq}} = 1036 \text{nH}$ and a resistance $R_{\text{eq}} \sim 9.8 \text{kΩ}$ were calculated. Thus, the final capacitance values were 356 pF for the tuning capacitor $C_t$, and 58 pF for each coupling capacitor $C_m$. Although their relative contributions differed greatly, the parasitic losses for both coils were of the same order.

**Figure 10.** Proportions of the various losses in: (a) the circular loop coil; (b) the solenoid-shaped coil.
Short Report

Non-invasive 3D time-of-flight imaging technique for tumour volume assessment in subcutaneous models

JA Delgado San Martin1,2, P Worthington3 and JWT Yates1

Abstract

Subcutaneous tumour xenograft volumes are generally measured using callipers. This method is susceptible to inter- and intra-observer variability and systematic inaccuracies. Non-invasive 3D measurement using ultrasound and magnetic resonance imaging (MRI) have been considered, but require immobilization of the animal. An infrared-based 3D time-of-flight (3DToF) camera was used to acquire a depth map of tumour-bearing mice. A semi-automatic algorithm based on parametric surfaces was applied to estimate tumour volume. Four clay mouse models and 18 tumour-bearing mice were assessed using callipers (applying both prolate spheroid and ellipsoid models) and 3DToF methods, and validated using tumour weight. Inter-experimentalist variability could be up to 25% in the calliper method. Experimental results demonstrated good consistency and relatively low error rates for the 3DToF method, in contrast to biased overestimation using callipers. Accuracy is currently limited by camera performance; however, we anticipate the next generation 3DToF cameras will be able to support the development of a practical system. Here, we describe an initial proof of concept for a non-invasive, non-immobilized, morphology-independent, economical and potentially more precise tumour volume assessment technique. This affordable technique should maximize the datapoints per animal, by reducing the numbers required in experiments and reduce their distress.

Keywords

xenograft, non-invasive, reduction, infrared

In preclinical oncology studies, subcutaneous tumour models are used to investigate the pharmacology of anti-cancer agents. These animal models rely on an estimation of the tumour volume, which can be estimated visually. Two methods of assessing tumour volume are typically used: calliper and in vivo imaging-based methods. In vivo imaging-based methods are generally more accurate than calliper measurements,1-3 but are laborious and dependent upon animal immobilization. Calliper methods are widely used, but rely on the measurement of characteristic lengths (length, width and/or height). In an effort to improve accuracy, 3D visual scanners demonstrate increased accuracy, and are minimally disruptive to the animals, but have limitations with regard to speed of image acquisition and animal manipulation.4,5 We propose the first stages of an off-the-shelf technique, which should overcome the problems of implementation observed until now, reducing both the number of animals needed and their distress.

Animals

Eighteen tumour-bearing NSG mice were used from several pre-existing experiments. No extra animals were employed specifically for this study. The mice

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were selected to reflect diverse morphologies and sizes. The methods of animal handling, maintenance and euthanasia (by cervical dislocation) were compliant with UK Home Office legislation, the Animal Scientific Procedures Act 1986 (ASPA), and with the AstraZeneca Global Bioethics policy. All experimental work was outlined in project licence 40/3483 which had gone through the AstraZeneca Ethical Review Process.

Materials and methods

Three calliper measurements were taken post-necropsy: width \( w \), length \( l \) and depth \( d \). The tumour is assumed to be either ellipsoid \((V = w \cdot l \cdot d/2)\) or spheroid \((V = w^2 \cdot l/2)\).

We used an off-the-shelf 3D time-of-flight (3DToF) camera (SoftKinetic DepthSenseDS325 Professional; SoftKinetic, Brussels, Belgium). This device provides both a visual HD720p image and a 240 × 320 pixel depth map. The camera captures continuous frames at 25 fps. We then randomly capture six frames and calculate their arithmetic rolling average. The method was calibrated using four clay mice with known tumour volumes.

Results

Inter-experimentalist variability introduces significant error. Three different expert experimentalists measured the same tumours with ellipsoid and spheroid methods.

Figure 1a presents the data in relative deviation from the mean:

\[ E_R(\%) = \frac{\sigma}{\mu} \cdot 100 \]

where \( \sigma \) and \( \mu \) are the standard deviation and mean.

Deviations from the mean for the regularly-shaped clay tumours were between 3% and 14% for calliper methods. The clay mouse showed a narrower deviation from the mean (1.8%), consistent with the fact that the tumour was close to being spherical.

Experiments: calliper versus 3DToF

For each mouse, three characteristic lengths of tumours were measured using an electronic calliper. The mice were then sacrificed and imaged using the 3DToF camera. The tumours were then excised and weighed as part of the normal protocol of the study.

Semi-automated image analysis was applied to the 3DToF-acquired depth maps. The camera was designed to detect hand gestures and we found the depth maps it generated to be very noisy.

The area of the depth map corresponding to the mouse flank was manually cropped and the tumour region was outlined to distinguish it from the background (i.e. the animal’s flank). A parametric quadratic surface of the type: \( z = a + b \cdot x + c \cdot y + d \cdot x^2 + e \cdot y^2 + f \cdot x \cdot y \) was fitted to the background depth points to provide a smooth model of the animal’s flank. We had experimented with a similar fitting
process for the tumour region, but the smaller number of datapoints affected the quality of the fit. Instead, we applied a low-pass Gaussian filter with $\sigma = 1$ to the raw depth map data in the tumour region. Figure 1b and 1c illustrate the resulting surfaces for a clay and real tumour, respectively. The tumour volume is calculated using the difference between the modelled flank surface (mouse skin) and the smoothed tumour surface, hence the volumetric integral could be calculated as the sum of all depths multiplied by the pixel area:

$$V_{ToF} = \int_{Surface} A_p \cdot \sum (H_{tum,i} - H_{skin,i})$$

where $A_p$ is the area of a pixel, $H_{tum}$ and $H_{skin}$ are respectively the height of the depth map at each point of the tumour surface.

Hand-made clay mouse models were used as a basis for the calibration and parameterization of the camera and method. These had tumours with regular shapes and slightly exaggerated sizes of $1–2\,\text{cm}^3$. The results using the 3DToF method compared with caliper measurements are presented in Figure 2a. Error is calculated relative to the true volume as measured using weight:

$$E(\%) = 100 \cdot (V - V_{\text{Weight}}) / V_{\text{Weight}}$$

The spheroid model consistently overestimates the volume, returning relative errors of $+30$ to $+160\%$. The ellipsoid measure presents a slightly narrower error window but again with a tendency to overestimate the volume in the case of the model tumours. Measurements based on 3DToF demonstrated similar but slightly improved accuracy to the ellipsoid method.

Tumour volume measurements in real mice are plotted against weight in Figure 2b. Three density lines are also shown (covering all physiological densities observed in mammals, $0.9\,\text{g/mL}$ [fat] and $1.06\,\text{g/mL}$ [plasma]). We assumed $1\,\text{g/cm}^3$ as our baseline measure. Figure 2b contains linear fits with a forced zero $y$-intercept of the datapoints with slopes of $0.63$, $0.96$ and $1.15\,\text{g/cm}^3$ ($37\%$, $4\%$, $-15\%$ of error) for spheroid, ellipsoid and 3DToF methods, respectively.

Tumour volumes studied range from as small as $0.2\,\text{g}$ (treated tumours) to $0.8\,\text{g}$ (control tumours), which conforms to the calliper confident detection range. Our studies should therefore be representative of any pharmacological study. The spheroid method is quite accurate with small tumours (in earlier development they are mostly spherical). However, larger tumours show a bias, with errors of up to $+14\%$. We infer from this that large tumours tend to be flatter rather than spheroidal, which is consistent with visual inspection of the tumours. On the other hand, the ellipsoid method is more accurate, with error rates mostly below $+34\%$ and within the range of possible densities. Finally, 3DToF presents a distribution either side of the $1\,\text{g/cm}^3$ line. Although for very small tumour sizes the 3DToF surface data are too noisy and lead to a scattered distribution, the overall trend leads towards a relatively accurate slope value.

The circled point on Figure 2b represents a clear outlier. From examination of the depth images, this datapoint corresponds to a particularly noisy depth map returning invalid outputs (not-a-numbers, NaN), where the mouse was too close to the device, and lying beyond the detection confidence dictated by the camera software developer’s kit (SDK).

In general terms, calliper methods overestimate tumour volume, whereas the 3DToF approach when using the low-cost depth camera currently underestimates it slightly.

**Figure 2.** Tumour volume results. (a) Results of volume estimation error (y-axis) of the 4 clay mice (x-axis) for the 2 calliper methods and 3DToF. (b) Results of animal models. Solid lines represent ranges of human physiological densities (tumour xenografts should be between them). Dashed/dotted lines are fits of the 3 methods. Markers are individual measurements.
Although the ellipsoid method outperforms the current 3DToF, we believe our approach shows sufficient promise to warrant further investigation. The 3DToF technique as described suffers from noisy depth data. However, there are much more accurate low-cost 3D cameras currently entering the market (e.g. Fuel 3D, http://www.fuel-3d.com/) which will substantially improve accuracy.

Due to the high levels of noise, the animal’s flank and the tumour outline are currently estimated by manual tracing, which introduces an element of human variability. Development of a robust automated technique for this stage of the analysis would be needed for the effective practical application of the technique.

Significant further development would be needed in order to be able to obtain tumour measurements in a live mouse, particularly one that is free to roam.

**Conclusion**

The 3DToF method represents an initial demonstration of the concept that a non-invasive, low-cost and potentially more precise method based on off-the-shelf technology could be developed. Such a method should enhance experimental sensitivity by minimizing both experimentalist error and systematic inaccuracy (see supplementary material which can be found online with this article at http://lan.sagepub.com). The camera could be installed online or on the scale, automatically registering both weight and volume. More accurate measurements, including track of the morphology, would thereby be obtained daily. This would multiply time points, which would improve the statistical power of the tumour growth data, requiring the use of fewer animals to identify mathematical models. At the same time, visual image analysis could be used to identify external signs (such as rashes or ulceration). In summary, more sophisticated hardware combined with advanced image analysis could provide a non-disruptive, robust and accurate method for subcutaneous tumour volume measurement, which would significantly reduce the number of animals needed.

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**Declaration of conflicting interests**

The authors disclose no potential conflicts of interest.

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Surgical procedure of extracting teeth for obtaining dental pulp for regenerative medicine in swine

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Abstract
Dental pulp is a potential source of cells that can be used in cell replacement therapy for various nerve disorders, including stroke, spinal cord injury, and peripheral nerve defect. However, the validation of an animal model closely related to humans is needed in translational research. The miniature pig is a suitable experimental model in maxillofacial surgery, because its anatomical structure and size are similar to those of humans. However, the swine tooth is extremely long. The routine closed extraction procedure for harvesting dental pulp tissue causes root fracture. This report describes the details of a surgical procedure for tooth extraction. Four healthy 7–8-month-old male NIBS miniature pigs were used. Two mandibular deciduous right incisors (Di1 and Di2) were extracted in order to obtain dental pulp tissue. Gingival envelope incision with vertical-releasing incision was performed, and a full-thickness mucoperiosteal flap was made. The buccal alveolar bone was exposed and removed by osteotomy. Di1 and Di2 were extracted. Dental pulp tissue was obtained from these extracted teeth by splitting hard tissue. In this procedure, 9.8 ± 2.5 × 10⁵ cells were obtained from the mandibular Di1 and Di2 (n = 4).

Keywords
dental pulp, miniature swine, tooth extraction, experimental model

Dental pulp is a potential source of cells that can be used in cell replacement therapy for various nerve disorders, including stroke, spinal cord injury, and peripheral nerve defect. Dental pulp derived from the adult human third molar contains cells that are clonogenic and highly proliferative. Adult human dental pulp cells are also capable of differentiating into adipocytes and neuron-like cells. Moreover, in generating induced pluripotent stem cells, dental pulp cells may be stored in cell banks and used for regenerative medicine. However, to achieve this outcome, the validation of an animal model more closely related to humans is needed in translational research. Miniature pigs are a suitable experimental model in translational research, (1) because of their anatomical structure, and (2) because their size is similar to that of humans. However, no report is available which describes the details of surgical procedure of dental pulp collection for translational research using miniature swine. Without this information, difficulties arise in extracting teeth, because the root of swine teeth is extremely long, and the routine closed extraction procedure causes root fracture. Therefore, this report describes the details of a surgical procedure for tooth extraction under intravenous anaesthesia.

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Materials and methods

Four healthy 7–8-month-old male NIBS miniature pigs (12.2–21.9 kg) (Nisseiken, Ome, Japan) were used for this study. Mandibular deciduous right incisors 1 and 2 (Di1 and Di2) were extracted to obtain dental pulp tissue. Animal care and handling procedures were performed in accordance with the ‘Principles of Laboratory Animal Care’ of the Tokyo Women’s Medical University Animal Experimentation Committee. Harvested dental pulp cells were used for the autologous peripheral nerve regeneration study. The pigs were given enrofloxacin at a dose of 5 mg/kg by subcutaneous injection after surgery and fasted for a minimum of 12 h after surgery. The pigs were given only pain-relief via local anaesthesia, because they underwent further surgery in an autologous peripheral nerve regeneration study several hours after the extraction of their teeth. The pigs were sacrificed at the end of this project by an intravenous injection of potassium chloride under sevoflurane-induced anaesthesia. No adverse effects were observed.

Surgical procedure

- The miniature pig was first premedicated by a combined intramuscular injection of medetomidine (80 μg/kg), ketamine (5 mg/kg), and butorphanol (0.2 mg/kg), and then anaesthetized by an intravenous injection of 1% propofol (induction: 20–30 mg), and maintained by repeated intravenous injections of 10 mg propofol that were given every 10 min.
- Because the root of the swine incisor is extremely long, routine closed extraction caused root fracture. Therefore, an open extraction was needed. A full-thickness mucoperiosteal flap was created on the buccal side of the gingiva (Figure 1a). The flap included the surface mucosa, submucosa and periosteum. The base of the flap had to be broader than the free margin to preserve an adequate blood supply. A narrow flap at the base might have led to flap necrosis. The margin of flap had to be located on the intact bone that would remain after the surgical procedure was complete in order to promote healing.12
- Local anaesthesia was injected into the subperiosteal using 1% lidocaine HCl and hydrochloride epinephrine at a dilution of 1:100,000 through a 23-gauge needle for (1) inducing local analgesia, (2) reducing the amount of haemorrhage during extraction, and (3) facilitating subperiosteal dissection by hydrodissection.
- A gingival envelope or sulcular incision with vertical-releasing incision was performed using a No. 15 scalpel, and a trapezoidal full-thickness mucoperiosteal flap was made. The alveolar bone was exposed by peeling the subperiosteal with a periosteal elevator. The mental nerve was observed at the alveolar bone of the apical region (Figure 1b).
- The alveolar bone of the buccal root surface was removed by green stick fracture, which was performed by osteotomy using a bone chisel and hammer. Osteotomy was performed at (1) the buccal surface of the root of Di1 and Di2, (2) the distal of root surface of Di2, and (3) the medial of the root surface of Di1. The buccal surface of the root of Di1 and Di2 was then exposed (Figure 1c).
- Each tooth was extracted using dental extraction forceps. The crown and root were grasped as deeply as possible, and luxated by shaking the tooth, which was removed toward the buccal side (Figure 1d).
- When the green stick fractured alveolar bone and mucoperiosteal flap were repositioned, the wound was sutured by simple suture with 3–0 degradable surgical sutures. If any free alveolar bone was observed, it should be removed to prevent bone necrosis. The first suture was placed on the papilla of vertical-releasing incision, the second suture was placed on the other papilla, the third suture was placed on the socket, and finally the vertical incision line was sutured (Figures 1e and 1f).
- Dental pulp tissue was split and collected from the extracted teeth by splitting using bone cutting forceps or bone rongeurs (Figure 2).

Dental pulp cell harvest

Dental pulp cells were harvested using a previously reported method with slight modifications to the preparation of periodontal ligament cells.13 Briefly, the dental pulp tissue was mechanically minced and treated with a solution of collagenase type I (SERVA Electrophoresis, Heidelberg, Germany) with 10,000 PU/mL dispase (Eidia, Tokyo, Japan) in α-MEM glutamax (Gibco, Carlsbad, CA, USA) for 2 h at 37°C with vigorous shaking. Single-cell suspensions were obtained by passing the cells through a 70 μm strainer (BD Falcon, Franklin Lakes, NJ, USA). The disaggregated cells were counted, and the data were expressed as the mean values ± standard deviation (SD).

Results

A total 9.8 ± 2.5 × 10^5 dental pulp cells were obtained from the two mandibular deciduous incisors using this procedures \((n = 4)\). All the experimental procedures
were found to have minor complications such as facial swelling and non-active bleeding.

Discussion

In cell replacement therapies for various nerve disorders with tissue engineering, autologous transplantation is better than allograft, because allograft causes immune rejection, and the patients need immunosuppressants throughout their lives. Therefore, the cell source should be easily accessible with a low complication for autologous transplantation. Dental pulp, derived from neural crest, contains Schwann and stem cells. The third molar is an easily accessible source of human dental pulp. The extraction of the third molar is one of the most common basic procedures performed in an oral and maxillofacial surgery unit. The overall complication
The rate is low, and most complications are minor. The extracted third molar is routinely discarded as medical waste. Previously, the authors have isolated rat dental pulp cells by an enzymatic method and have incorporated them into collagen gel, which could then be placed into a nerve guide. A nerve guide containing collagen gel with dental pulp cells has been found to regenerate nerve cells more effectively than the control nerve guides containing the gel alone. Furthermore, transplanted dental pulp cells contain dental pulp-derived endothelial cells and Schwann cell-like supportive cells. These results suggest that axon and blood vessel regenerations are promoted by the dental pulp-derived supportive cells, which are produced by neurotrophic factors, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and glial cell line-derived neurotrophic factor (GDNF).

Miniature swine can be used as an experimental or pre-clinical model in maxillofacial surgery, including mandibular reconstruction and distraction osteogenesis, dental implantology, bisphosphonate-related jaw osteonecrosis, and face transplantation. In this study, Di1 and Di2 were used for harvesting dental pulp tissue, because (1) mandibular incisors can be better accessed than molars, (2) a single root incisor can be easier to extract than the molar which has four or six roots, and (3) Di1 and Di2 have a larger dental pulp than Di3 or Dc1 at the age of 7–8 months. Di1 and Di2 are erupted at 1–3 weeks and 2–3 months, respectively, whereas permanent I1 and I2 are erupted only at 12–16 and 16–20 months, respectively. All incisors in miniature pigs have a very short crown and a long simple root, and their size is similar to that of humans, and because they are suitable for surgical training and for teaching new innovative transplantation methods.

This report outlines a surgical procedure of tooth extraction in swine and using dental pulp as a cell source for autologous cell transplantation by regenerative medicine. The miniature pig has both deciduous and permanent dentitions, which are similar to those of humans. The dental formulae of the deciduous dentitions are Di3/3, Dc1/1, Dp2/3, Dm2/1, and those of the permanent dentitions are I3, C1, P4 (3), M3 (maxillary) and I3, C1, P3 (4), M3 (mandible). In this study, Di1 and Di2 were used for harvesting dental pulp tissue, because (1) mandibular incisors can be better accessed than molars, (2) a single root incisor can be easier to extract than the molar which has four or six roots, and (3) Di1 and Di2 have a larger dental pulp than Di3 or Dc1 at the age of 7–8 months. Di1 and Di2 are erupted at 1–3 weeks and 2–3 months, respectively, whereas permanent I1 and I2 are erupted only at 12–16 and 16–20 months, respectively. All incisors in miniature pigs have a very short crown and a long simple root, and
the length of the root is two to three times than that of the crown.\textsuperscript{11} Therefore, an open extraction is needed to prevent root fracture. It was possible to harvest approximately $10 \times 10^5$ dental pulp cells from the mandibular incisors Di1 and Di2. Although the required number of dental pulp cells for regenerative medicine is still unclear, the authors have previously reported that $1.0 \times 10^5$ transplanted dental pulp cells can regenerate a 7 mm nerve gap in a rat model.\textsuperscript{3} If a larger amount of dental pulp cell is needed, a bilateral Di2 could be extracted and a vertical-releasing incision could be made bilaterally distal to the Di2. Our method could contribute toward the progress of regeneration research using dental pulp tissue in miniature swine.

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Declaration of conflicting interests

The authors declare no conflicts of interest. R Sasaki holds the position of Assistant Professor at the Tokyo Women’s Medical University.

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Calendar of events

Meetings of interest to laboratory animals scientists and technicians: references to Laboratory Animals are for further details. Items for inclusion should be sent to Notes and Comments Editor, Laboratory Animals, Charles Darwin House, 12 Roger Street, London WC1N 2JU, UK. Email to comments@lal.org.uk. The deadlines for inclusion of material are: January issue, 10 October; April issue, 10 January; July issue, 10 April; October issue, 10 July.

2015

8 April Establishment Licence Holders’ Forum Spring Meeting, London. For further information visit info@lasa.co.uk
21 April LASA/LAVA Severity Assessment Meeting, Cambridge, England. For further information visit info@lasa.co.uk
3–5 June AFSTAL Annual Scientific Meeting, Lille, France. For further information visit http://www.colloque-afstal.com/2015/
9–12 June Scand-LAS Annual Scientific Meeting, Turku, Finland. For further information visit www.scandlas.org
16–19 June Genetics, breeding and welfare of laboratory mice, Cambridge, England. For further information visit https://registration.hinxton.wellcome.ac.uk/display_info.asp?id=445
19 June LASA/London School of Hygiene and Tropical Medicine, Creature comforts, London. For further information visit info@lasa.co.uk
19–22 July European Association for Veterinary Pharmacology and Toxicology (EAVPT) and the European College of Veterinary Pharmacology and Toxicology (ECVPT), 13thEAVPT International Congress, Nantes, France. For further information visit www.alphavisa.com/eavpt/2015/
14–16 September GV-SOLAS Annual Scientific Meeting, Hannover, Germany. For further information visit: http://www.gv-solas.de/
30 September–1 October LAVA annual meeting, Cardiff, Wales. For further information visit http://www.lava.uk.net/
1–5 November AALAS National meeting, Phoenix, Arizona. For further information visit http://nationalmeetingaalas.org/future_sites.asp
18–20 November SECAL-SPCAL annual congress, Caceres, Spain. For further information visit www.secal-spcal2015.com
24–25 November SGV annual meeting, Lausanne, Switzerland. For further information visit www.sgv.org
25–27 November LASA Winter meeting, south coast, UK. For further information visit info@lasa.co.uk

2016


Index to Advertisers

<table>
<thead>
<tr>
<th>April 2015</th>
</tr>
</thead>
<tbody>
<tr>
<td>3Rs Lab   xvi</td>
</tr>
<tr>
<td>Altromin International  OBC</td>
</tr>
<tr>
<td>AnLab Ltd   xxv</td>
</tr>
<tr>
<td>Bell Isolation Systems Ltd   xxii</td>
</tr>
<tr>
<td>Charles River Laboratories   IFC</td>
</tr>
<tr>
<td>Datesand Ltd   xvi</td>
</tr>
<tr>
<td>Dustcontrol AB   xxiii</td>
</tr>
<tr>
<td>Edstrom Inc      x</td>
</tr>
<tr>
<td>Fine Science Tools GmbH   iv</td>
</tr>
<tr>
<td>Fine Science Tools GmbH   xx</td>
</tr>
<tr>
<td>GVG Genetic Monitoring   xiii</td>
</tr>
<tr>
<td>Harlan Laboratories   xxiv</td>
</tr>
<tr>
<td>Indulab AG   xxii</td>
</tr>
<tr>
<td>Institute of Animal Technology   xxvi, xxvii</td>
</tr>
<tr>
<td>IPS Product Supplies Ltd   xxi</td>
</tr>
<tr>
<td>Laboratory of Pharmacology and Toxicology GmbH &amp; Co KG   vii</td>
</tr>
<tr>
<td>LBS   xviii</td>
</tr>
<tr>
<td>Marshall BioResources   xix</td>
</tr>
<tr>
<td>NuAire Inc   viii</td>
</tr>
<tr>
<td>PFI Systems Ltd   xii</td>
</tr>
<tr>
<td>R C Hartelust bv   xxv</td>
</tr>
<tr>
<td>SAFE   xv</td>
</tr>
<tr>
<td>Special Diets Services   xvii</td>
</tr>
<tr>
<td>Ssniff Spezialdiäten GmbH   ix</td>
</tr>
<tr>
<td>Sychem   xxviii</td>
</tr>
<tr>
<td>Tecniplast SpA   iii</td>
</tr>
<tr>
<td>Tecniplast SpA   IBC</td>
</tr>
<tr>
<td>Vet-Tech Solutions Ltd   xi</td>
</tr>
</tbody>
</table>
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