Ethical Use of Animal Models in Musculoskeletal Research

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1. Abstract

The use of animals in research is under increasing scrutiny from the general public, funding agencies and regulatory authorities. Our ability to continue to perform in-vivo studies in laboratory animals will be critically determined by how researchers respond to this new reality. This Perspectives article summarizes recent and ongoing initiatives within ORS and allied organizations to ensure that musculoskeletal research is performed to the highest ethical standards. It goes on to present an overview of the practical application of the 3Rs (reduction, refinement and replacement) into experimental design and execution, and discusses recent guidance with regard to improvements in the way in which animal data are reported in publications. The overarching goal of this review is to challenge the status quo, to highlight the absolute interdependence between animal welfare and rigorous science, and to provide practical recommendations and resources to allow clinicians and scientists to optimize the ways in which they undertake preclinical studies involving animals.

Keywords: preclinical; in vivo; 3Rs; ethics; veterinary clinical trials; orthopaedic; review

2. Introduction

The use of laboratory animals remains a critical step in the preclinical evaluation of pharmaceuticals, biologics and biomedical devices. There is increasing public opposition to the use of laboratory animals, and our ability to continue to use animals in research will critically depend on how the field responds to this changing conversation. For many, whether veterinarians, physicians or scientists, the arguments made against the use of animals resonate, and while we may continue to support the use of animals in research, each of us has a particular comfort level regarding what is or is not justifiable in the name of medical research. Independent of this individual view there is no place in science for ill-designed, poorly executed and inadequately reported studies of any type. When these are cell culture studies, they are financially and scientifically unjustifiable; if they involve animals, they are also ethically unsound, unacceptable and need to be stopped. It is our contention that the best approach to addressing the public's concerns over the use of animals in research is to ensure that the scientific community works collectively to regulate itself, and that the steps that are being taken to maintain the highest ethical standards are both transparent and consistent.

One of the most significant obstacles to improving the quality of animal research is the lack of uniformity in the training that researchers receive before they start their research careers. This is then compounded by significant variability in the financial and technical resources, including infrastructures, that are available to them on a daily basis. Over the next couple of years, this journal will partner with topic experts to produce a series of "best practice" articles that will drill down into the specifics of some of the core research areas in which animal models play a central role. In parallel, the Orthopaedic Research Society (ORS) will provide a new forum for researchers using animal models in their research. The goal of the new 'Preclinical Models' section (http://www.ors.org/preclinical/) is to help the Society's members design and perform animal studies to the highest ethical and scientific standards.

This *Perspectives* article seeks to summarize recent ORS initiatives relating to the use of animals in preclinical research, and to present an overview of the key issues that need to be considered when planning animal studies related to musculoskeletal research. The

goal is not to provide a complete how-to guide, rather to stimulate the reader's curiosity. Much of what we do as researchers is done because "*that is how we were told to do it*." When it comes to the use of animals, we are duty bound to challenge the status quo and to critically assess our methodology. By providing new tools to educate researchers about alternatives to using animals, and by training them on new techniques to improve animal study design and technical competence, we hope to fuel a grass-roots process that will lead to substantive improvements in both the quality and the ethical standards of animal studies in musculoskeletal research worldwide.

3. ORS Initiatives Relating to the Use of Animals in Musculoskeletal Research

The ORS has long realized the importance of animal models in the research that its members undertake. Presentations on animal studies can be seen across almost every research theme that comes under the ORS umbrella, both at the Annual Meeting and in this journal. Over the last 5 years, there has been a growing demand from the membership for improvements in the way that this work is conducted and, in particular, presented. With a global membership, ORS attracts researchers from many nations, and the regulatory procedures relating to research involving animals vary widely. While it is not the purview of the ORS or any other society to dictate the means through which countries regulate animal studies, it is entirely appropriate for the Society to expect its members, as well as non-members who want to present work at our meetings, to ensure that appropriate steps are taken to prevent unnecessary pain, suffering or distress, and to follow the central tenet's of the 3Rs of animal research – reduction, refinement and replacement. With this in mind, the Journal of Orthopaedic Research has now adopted the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines[1] as part of the manuscript submission process; this will ensure that researchers understand and commit to the guiding principles of ethical animal use, and that their methods are clearly reported. The expectation is that by requiring researchers to formally commit to meeting ARRIVE requirements, the Society will change the way that researchers approach their research. At the same time the Society will provide enhanced educational content (through "best practice" papers and through the educational offerings of the Preclinical Models section) to equip researchers with the tools to be the agents of change themselves, without the need for changes in the

national regulatory processes. Ultimately, the use of animals in research is a privilege, and like all privileges it comes with responsibility. We are the stewards of the animals that we use in research, and we are ethically bound to ensure that the procedures that are performed on these animals are justifiable, ethical, performed by individuals with appropriate technical skills, and backed up with clinically proven anesthesia and analgesic protocols to alleviate unnecessary pain or distress.

Veterinarians and others interested in animal models developed an "animal models" research interest group (RIG), and held sessions at both the 2015 and 2016 Annual Meetings. In 2015, the RIG held an early morning session entitled "*Good and Bad Animal Models*" was organized and chaired by Dr. Stephan Zeiter of the AO Research Institute in Davos. Speakers included Dr. Christopher Little of University of Sydney and Dr. Karl Kirker-Head of Tufts University. A second session, a workshop in the main ORS program, was entitled "*Animal Welfare in Orthopaedic Research: Focus on Refinement and Reduction*" and coordinated by Mr. Tim Cooney, a research associate of the University of Pittsburgh Medical Center, Hamot and Dr. Laurie Goodrich of Colorado State University's College of Veterinary Medicine.

In 2016, the RIG focused on plans to develop an ORS section dedicated to discussions about animal use in musculoskeletal research. As a direct result of that discussion, and with the support of members at the RIG, plans were enacted to develop a new ORS section. The new '*Preclinical Models*' section is the third to be approved by the ORS Board of Directors, following the paths taken by the very successful '*Spine*' and '*Tendon*' sections. Initially, the section will meet at the Annual Meeting, but future plans include the development and deployment of educational content online, through symposia and at hands-on laboratories that will provide trainees and more senior researchers with cutting-edge skills for performing animal research. It is our hope and expectation that the *Preclinical Models* section will provide a resource to the entire scientific community - a place where researchers can seek and offer advice, discuss the pros and cons of animal models for a particular research question, identify mentorship and training opportunities, and participate in seminars and laboratories to develop and hone new technical skills.

4. Evolution of the 3Rs and Implications for Musculoskeletal Research

In 1959 Russell and Burch published their seminal book on ethical experimentation.[2] Since then the use of the term the "3Rs", referring to the replacement, reduction and refinement of animal experimentation, has been the foundation for high quality and humane scientific research. Continued understanding and implementation of the 3Rs is essential for acceptance of orthopaedic studies using animals. There are multiple resources available to help in the understanding of alternatives for animal research. Organizations from around the world have an online presence that can help with the 3Rs and alternatives. A good site for locating centers is http://caat.jhsph.edu/resources/. The following is a brief introduction to the 3Rs.

Replacement is the most commonly cited of the 3Rs. The goal of replacement is to use alternatives to animals in research whenever possible. Examples include the inclusion of human volunteers, tissues and cells, mathematical and computer models, using established animal cell lines, invertebrates, or immature forms of vertebrates. An example of the replacement of animal models is the development of a robotic manipulator to simulate clinical tests and gait on cadaveric joints.[3] In this study, joint biomechanics were first defined in human subjects and then modelled in a robotic simulator. Similarly mathematical models generated from in-vivo data can also be used for studying mechanical force patterns. Finite element modelling of bone, for example, has been used to explore the cause of hip injuries.[4] The successful integration of mathematical or robotic models depends on the availability of valid data to inform the model, and these necessarily come from animals or humans in the first instance. The real strength of computational models lies in their use for parametric studies, where the goal is to isolate single variables (e.g. to study the influence of pre-tensioning on the behaviour of ACL grafts). For more complex studies, especially those involving biological processes such as healing or tissue remodelling, it is impossible to replicate the in vivo environment and animal studies of some type are still needed.

Another area of important advancement in replacement is the use of less sentient species. Zebrafish and insects are rapidly expanding the horizon for research models in multiple fields. This includes orthopaedic research involving tendon, muscle, and bone.[5-

[7] Conceptually the replacement of a mammalian species with fish and insects would lead to less pain and distress and thus is considered to be more humane, although it should be noted that we understand little of pain perception in fish and insects at the current time, making this an topic of ongoing debate.

Reduction, the second R, seeks to minimize the number of animals used in an experiment. This typically relies on statistical analysis to justify the number of animals used in the experiment. Although difficult to estimate for many studies, there is enough historical data on experimental variables that determining the power of an experiment should be readily achievable. It is important to be mindful of the fact that power calculations have limited validity – they relate to a specific model and to a specific outcome measure. This underpins the importance of researchers fully disclosing their methods. If complete details are provided, other researchers can duplicate the test methods and use the existing data to support a new power analysis. Reduction of animals can also include sharing of animals between research groups. An example of this is the joint publications examining the outcome of high fat diet and exercise on a variety of systems. The primary investigator was interested in renal disease associated with diabetes, and was willing to share the musculoskeletal system for use by another investigator.[8, 9] This sharing of tissues halved the number of animals required if the studies would have been performed separately. Investigators should take full advantage of these and similar opportunities.

Refinement, the third R, refers to strategies designed to minimize the pain, suffering, or distress experienced by animals. In orthopaedic research, surgical interventions are common and can most frequently benefit from refinements. Refinement can be enacted at multiple steps of the process – by ensuring that the surgical team is technically proficient in the procedure that is to be employed; by having trained personnel assessing animals in the post-operative period; and through the mandatory use of proven anesthetic and analgesic agents to control post-procedural pain. One frequently articulated concern of the research teams is that addition of an analgesic during a procedure may alter the biologic process that is being studied. However, pain and

distress also can lead to alterations in healing responses,[10] compromising the quality of the science. It is our position that analgesics of some form should **always** be administered when invasive procedures are performed on animals. When considering the use of analgesics, investigators should be aware of the current use in humans so as to best model possible translational opportunities.

The need to improve the design, conduct and analysis of research using animals is an ongoing process, with increasing emphasis from the research community on improving animal welfare. It is interesting to speculate on the future of the 3R's as the need for sound scientific research is just as relevant today as it was nearly 60 years ago. Since the initial description there have been two additional "R's" that are being proposed as essential components of high quality animal research studies. These are **Responsibility** and **Reproducibility**. Responsibility takes into account the new performance based outcomes that should reflect integrity, honesty, and scientific correctness in appropriate and reasonable use of laboratory animals.[11] Reproducibility of research results relates to a topic that is touched on earlier in this review – the notion that it is impossible to make valid comparisons between studies when the methods used to derive the data have not been documented appropriately. Irreproducibility in animal work is inconsistent with the tenets of the 3Rs, since we are clearly unable to avoid unnecessary duplication of work, let alone ensure reductions in animal use.[12] It is also fiscally unsound in an era of increasing pressure on research funds. It was recently estimated that irreproducibility in preclinical research wastes around $24 billion per year.[13]

5. Practical Recommendations for Integrating the 3Rs into Musculoskeletal Research

The re-emergence of the 3Rs as a fundamental guiding principle for animal research has led to the development of emphasis on the practical applications of these principles at every stage of design and execution of an animal study. In this section, we will review the practicalities of implementing the 3Rs in animal-based research and draw upon our collective experience to support this approach and explain why attention to the 3Rs is so important.

5.1. Experimental Design

The starting point when considering any experimental design is to understand the specific question that is being asked, and the most relevant outcome measures that are to be reported. Is the study intended as a proof of principle study to determine technical feasibility or biological activity, or is it a pivotal preclinical study for the purpose of regulatory submission? Have data been collected from animal models prior to this study? Where do the gaps in knowledge lie? The answers to these questions will impact the choice of animal, the complexity of the experimental matrix, and the selection of an appropriate sample size for the study. The choice of outcome measures will ideally be based on clinical and translational relevance, but will also be influenced by the availability of resources, the technical expertise of the research team, and the budget that is available. Non-invasive imaging, which plays such a critical role in clinical and preclinical orthopaedics, offers tremendous opportunities for both refinement and replacement of animals. However, the trade off is that the instrumentation can be expensive to purchase and maintain, while the interpretation of the large and typically complex datasets can be technically challenging and time consuming

One of the core elements of the ethical review process is the avoidance of **unnecessary** duplication, but some degree of duplication will often be necessary in order to ensure relevance and validity of the data. Pilot studies, for example, are intended to develop preliminary data and methods; the same overall experimental design may then be used for a larger follow-up study that will expand on the early data and provide appropriate statistical power for data analysis. Duplication of an existing technique is both necessary and to be encouraged in most cases since the use of an accepted and well characterized animal model will help to reduce the problem of irreproducibility that currently complicates the interpretation of animal studies from different laboratories and different countries. Over time, the adoption of standardized methodologies and improved reporting mechanisms will make it easier to compare the results from new therapy against those from therapies that have already been evaluated in the same model. This will improve the accuracy of sample size calculations (which are commonly based on published data) and, as importantly, it may make it possible to reduce overall animal use since comparisons could be made against historical data from earlier studies.

The best approach to avoiding duplication is to remain current with the latest developments in the field of research, through the reading of the latest research articles and attendance at meetings. A detailed and up to date literature review should be performed, not just to meet the requirements of the ethical review process but also to challenge and encourage the researcher to consider refinements in the experimental technique, perhaps through the use of cutting-edge imaging. In addition, with the explosion of online resources and social media networks, it is easier then ever to connect to other investigators in the field and to ask for advice regarding model selection. As an example, the veterinary division of AO (www.aovet.aofoundation.org) has recently launched an initiative to develop an online, searchable database for orthopaedic animal models [Kirker-Head C, personal communication]. In the long term, care taken at this early point in study development will help to ensure that the model selection and technical procedures are acceptable to the research community and less likely to encounter challenges as they come to peer review for presentation and publication.

5.2. The Pilot Study

It is impossible to overstate the benefits of a pilot study and its potential positive impact on the quality of the final research product. Pilot studies provide an opportunity to evaluate every aspect of the study, from anesthesia and surgery, through post-operative care to the collection of both *in-life* and *post-mortem* endpoint data. The benefits are perhaps most obvious when performing complex procedures or experiments where a multidisciplinary team may need to learn to function efficiently together. However, the impact can be equally significant in experiments where a new drug is being evaluated in an established model, offering an opportunity to refine and validate standard operating procedures for drug preparation, administration and the identification of anticipated or unanticipated treatment-related side effects. Many institutional animal care and use committees (IACUCs) actively encourage the use of pilot studies because of the likely benefits of practice in everything that we do, and because the inclusion of a pilot study signals the willingness of the investigators to evaluate, refine and confirm their procedures ahead of large-scale animal use.

5.3. Time Points and Outcome Measures

In most cases, the selection of time points for a specific experiment will be based on personal experience, published regulatory guidelines, or a review of previous published data. However, in instances where a new outcome measure is proposed, or in which the purpose of the study is more mechanistic than end-point based, it is extremely important to pay attention to the time points that are to be used. For example, studies on a new surface coating or drug therapy to enhance implant fixation may use an end-point to confirm overall efficacy, but mechanistic information can only come from the study of time points that reflect key biological stages in the healing process. It may well be that overall fixation of the implant is the same as with a predicate device or treatment, but if the rate of healing can be shown to increase, the new approach may well have clinical merit in that it will allow patients to return to function earlier. In plain terms, the destination may be the same, but if the journey is different then there can be clinical impact (positive or negative). Pilot studies can be extremely helpful in this regard, allowing for sampling of small numbers of animals at regular time points in order to develop descriptive (qualitative) data on the healing process. These data can then inform the selection of the most appropriate time points for a larger study that can provide objective data on the mechanisms underlying the different healing rate. By rationalizing the selection of time points and basing the choices on science rather than habit, it is usually possible to achieve significant reductions in overall animal use while maximizing the amount of information gleaned from individual animals. As one moves from animal to human, it is important to recognize that inter-species differences in tissue remodeling rates can significantly impact the translatability of preclinical findings,[14] but the expectation would be that mechanisms are more conserved between species.

The outcome measures used in preclinical animal studies can be broadly classified into those with a direct clinical equivalent (e.g. radiography, computed tomography, serum or urinary biomarkers, biopsy material) and those that are limited to preclinical research (e.g. gross anatomical analysis, mechanical testing. In general, the latter tend to be more invasive and/or destructive, while the former tend to be non-invasive or minimally invasive and, as a consequence, more feasible for use in human clinical. While it is hard

to make definitive recommendations in this area, early-phase studies in rodents and rabbits are more likely to make use of invasive testing and intermediate time points with terminal evaluations, while pivotal large animal studies are more commonly designed with end-points that reflect potential outcome measures that might be of clinical interest in early-stage human clinical trials.. The use of a parallel set of outcome measures in preclinical and clinical trials offers huge potential value in terms of enhancing the translational relevance of the preclinical work; for example, if preclinical animal studies can be used to define and validate the relationship between magnetic resonance imaging (MRI) appearance and histology for a new cartilage repair strategy, the MRI findings from future human clinical trials will be easier to interpret, allowing for the use of histopathology as a confirmatory rather than exploratory outcome measure.

5.4. Conducting the Study

With the move towards the era of large interdisciplinary research teams and "big science", there is a much greater emphasis on inter-disciplinary teamwork in research.[15] As a result, it is commonplace to see investigators from engineering, cell biology and medicine working together on an experimental study. There are clear benefits to the development of this team-based approach, but it also creates challenges, especially with regard to experience in, and attitudes towards, animal research. It is vital that the team discusses the logistics of working together on an animal study to ensure that everyone is on the same page with regard to experimental design and study conduct. Whenever possible, it can be extremely beneficial – we would argue that it should in fact be standard practice - to involve an experienced veterinarian as either a co-investigator or a consultant to provide input on best practices in drug administration, anesthesia and analgesia, post-operative care and euthanasia. It is usually very helpful to engage the institution's animal care staff by presenting an overview of the work, so that they can better understand the goals of the work, the potential for complications, and the steps that need to be taken to manage those complications.

Whether undertaken under *Good laboratory Practice* (GLP) guidelines[16] or not, it is important that every animal procedure is conducted under the umbrella of one or more standard operating procedures (SOP). Ideally, the SOPs should be developed following

consultation with individuals with prior training and experience with a given procedure; the draft SOP can then be evaluated and refined in a pilot study at your institution, and the definitive SOP is then used for all future studies. Deviations from the SOP should be recorded and reported when the work is presented and published (see below). Use of SOPs will reduce variation in procedural methodologies, reduce the number of animals needed to achieve statistical power for a given study design and decrease the risk of irreproducibility by ensuring that other groups can make use of the same experimental design.

5.5. Reporting the Study

As mentioned previously, the impact of any scientific study can be critically limited by deficiencies in experimental design and study execution, but it is often in the reporting of the work that the greatest deficiencies are seen. Whether by accident or intent, failure to accurately document experimental procedures, post-surgical complications and clinical outcome has a significant negative impact on the quality of the resulting manuscript. More importantly, it becomes impossible to repeat that experiment, or to relate the findings from that study to any other. Taken as a whole, failure to fully disclose the research methodologies significantly decreases the translational impact of the research because it is impossible for the reader to determine the relevance or the robustness of the science. For preclinical science to be relevant, it must be designed and conducted appropriately, but it must also be reported and disseminated in an efficient, timely and transparent manner. The publication of a set of recommendations regarding appropriate reporting of animal research, the ARRIVE guidelines[1] represent an important step in the right direction.

6. The Ethical Review Process

The ethical review of scientific research will always be a potentially contentious topic. While most if not all agree on the need for oversight, each of us brings personal experience and bias (conscious or unconscious) to discussions on this topic. Ethical review does necessarily delay researchers who want to be getting on with their experiments, but we would argue that appropriate and efficient ethical review is actually central to doing great science. If we are to make use of animals in our research, it is our

duty (not that of a committee) to ensure that we do so in an ethical and humane manner. The purpose of ethical review should be to provide external guidance to facilitate this, and of course to identify and block research that is inconsistent with ethical and humane principles. The review process therefore needs to be formative, timely, unbiased and based on current best practices. The committee charged with undertaking ethical review should be approachable, knowledgeable and responsive both to investigator needs and to changes in best practices in animal care, veterinary medicine and research methodologies. If ethical review functions in this way, it will be seen as being a valuable and important part of the process, not an obstacle that one must clear before being able to get on with the "real work".

Although the specific procedures for ethical review vary by country, the primary goal of the ethical review process should be to undertake a cost-benefit assessment to determine whether it is justifiable to make use of animals for a particular line of research. Additionally, steps need to be taken to ensure that investigators are appropriately trained and make use of procedures that minimize pain, distress and suffering as much as is practical while undertaking their research. The review process may be managed centrally by national agencies (such as the Home Office in the UK) or locally through institutional structures (such as the Institutional Animal Care and Use Committee in the US). Attempts at international harmonization are ongoing,[17] but until agreement has been reached it has been the policy of most publishers to accept data from animal studies as long as there is documentation of appropriate approvals from the relevant regulatory agency in the country in which the work was performed. This approach generally works well, but there continue to be cases in which authors fail to document key steps in the review and approval process, or in which the methods that have been approved in one country are inconsistent with best practices in another. Adoption of the Arrive guidelines by JOR will be beneficial in ensuring consistency.

7. Recent Initiatives to Increase Transparency in Animal Use

Two of the most significant developments in recent years have been the publication of consensus documents on the reporting of animal experiments (ARRIVE guidelines)[1] and the introduction of a framework for openness regarding animal testing in the United

Kingdom (the Concordat).[18] The implications of these documents are far-reaching, and while further changes are likely, especially with regard to the Concordat, there is hope that they will impact scientific research at a global level. The *Journal of Orthopaedic Research* will soon be implementing the ARRIVE guidelines into the review process for any manuscript that reports animal data, and a similar approach is likely to percolate down to abstract reviews for conferences. The additional steps required to comply with ARRIVE guidelines are not onerous but they provide a transparency that has to date been missing and that will significantly enhance the interpretability and impact and relevance of the published work. Importantly, the introduction of ARRIVE represents an important first step towards reducing the problem of irreproducibility that plagues science in general but animal models in particular.[12, 13]

8. New Strategies to Identify and Manage Pain and Distress in Laboratory Animals

8.1. Behavioral scoring/facial grimace for identification of pain

Research in musculoskeletal diseases often requires an intervention that has the potential to cause pain or distress in laboratory animals. Prevention, detection and relief of pain and distress are paramount in performing good scientific studies. This section will briefly outline some of the methodology for ensuring animal well being during orthopaedic studies.

It is generally understood, and required by regulatory agencies, that pain should be prevented or alleviated unless it is part of the scientific study, or it will jeopardize the research validity. In the latter case if the investigator is unable to relieve pain or distress then the patient should be euthanized. Given that most orthopaedic procedures are not examining pain per se, there is rarely a scientific justification for not providing routine analgesia, especially if it is administered consistently to all study animals as a matter of protocol. There are several analgesic substances available for prevention or treatment of pain. Historically the administration of opioids has been the primary treatment given to humans for pain prevention post-operatively. However, the development of new analgesics has led to the use of multimodal therapy to reduce the side effects of the high dose of opioids required for post-operative pain control. Multimodal analgesia is the use

of multiple agents to act synergistically for more effective pain control with fewer side effects than a single agent. There are several studies that have shown the improved efficacy of multimodal therapy. For example the recently approved intravenous formulation of acetaminophen (paracetamol) has been shown in combination with ketorolac (an NSAID) to improve post-operative pain control compared to either drug alone[19, 20] In clinical trials, two anticonvulsants, gabapentin and pregabalin, have also been shown to be efficacious in reducing post-operative morphine consumption with either reduced pain or similar pain levels.[21-23] Gabapentin type drugs bind to calcium channels in the spinal cord and brain thus reducing afferent excitatory activity. The use of these drugs also showed a reduction in side effects.[21-23] There are other multimodal analgesics that have been shown to improve pain relief, including TRPV1 agonists, NMDA receptor antagonists and alpha-2 agonists.[24] A review article further identified evidence supporting the use of multimodal analgesics for spine surgery. In it they suggested that there is good evidence that gabapentinoids, acetaminophen, neuraxial blockade and extended-release local anesthetics (in ascending order) reduce postoperative pain and narcotic requirements, fair evidence that preemptive analgesia and non-steroidal anti-inflammatory drugs (NSAID) result in reduced postoperative pain, and insufficient and/or conflicting (Grade I) evidence that muscle relaxants and ketamine provide a significant reduction in postoperative pain or narcotic usage.[25]

Another relatively recent improvement in pain control is the use of pre-emptive analgesia. Transmission of pain signals evoked by tissue damage leads to hyperalgesia, or sensitization, of the peripheral and central pain pathways. Pre-emptive analgesia is a treatment that is initiated before the surgical procedure to reduce this sensitization. The goal of pre-emptive analgesia is to stop pain before it starts, thus, preventing the physiological consequences of nociceptive transmission evoked hyperalgesia.[26, 27] Several clinical trials have been conducted to assess the impact of pre-emptive analgesia versus standard analgesic therapy. Unfortunately, despite the scientific rationale supporting pre-emptive analgesia, only NSAIDs have shown a positive effect for reducing post-operative pain compared to giving analgesics only post-operatively.[27, 28] Although no improvement in the short-term was shown with pre-emptive analgesia in all studies, there was no evidence that it was more painful. In addition, there is a paucity of

information on the control of long-term pain using pre-emptive analgesia. Use of pre-emptive analgesia is recommended especially in the context of using a multimodal analgesia.

One concern specific to musculoskeletal procedures relates to the possibility that NSAIDs may effect bone healing. A recent review article examining the clinical evidence for this showed that there are conflicting data on the validity of these results.[29] Another showed there is fair (Grade B) evidence that short-term use of NSAID result in no long-term reduction in bone healing or fusion rates.[25] As the use of NSAIDs is still being debated it should be approached cautiously, as it is imperative that the analgesic protocol does not affect the scientific results of the animal study. Even if NSAIDs are counter indicated another analgesic should be used to provide pain relief.

Current advances with sustained release formulations as well as topical analgesics have improved the potential for administration of analgesics to animals without causing handling distress. Examples including the topical use of fentanyl either in a patch or a gel (Recuvyra) show promise in long-term pain relief.[30] In addition, a single injection formulation of buprenorphine is available that provides 72 hours of pain relief.[31] A recent review of the available topical analgesics found that there are a number of new analgesics being developed.[32] It is clear from this work and the absence of a universal analgesic that future analgesic types and combinations are still needed to improve the post-operative welfare of patients.[33] As these are developed, transferring this information to our animal models is essential.

Although in human patients self-administration of analgesics is possible, for our experimental animal patients we are required to administer analgesics. Determination of pain is not easily performed because they are prey species that have an instinctive ability to disguise pain. Thus, it is often only subtle behaviors that will alert the investigative team of an animal in potential pain. Careful observation by the animal care staff is often the best method for daily assessments of animal well being, as the individuals who take care of the animals daily will have knowledge of what is normal behavior for the patient. To augment this assessment, though, there are a few

behavioral tests that have and are being developed to determine if there is evidence of pain. One of these is the use of nest building behavior to assess pain in mice.[34] This entails addition of a small amount of nesting material into the cage and evaluation of whether the mouse incorporates it into the nest. Mice in pain do not perform this task. Another assessment used in rodents and other species is the facial grimace score. Adapted from children, this scoring system allows assessment of pain based on facial features.[35, 36] A third potentially exciting new approach involves the use of whole-cage monitoring systems to quantify changes in activity patterns within group-housed animals.[37]

9. Objective Measures of Musculoskeletal Functional Recovery

In a review paper of this type, it is impossible to provide details on all aspects of objective evaluation of musculoskeletal function. This section will present an overview of some of the common outcome measures and some thoughts on how they may be usefully applied to preclinical studies. The main application of the 3Rs in this context is the reduction of animal numbers as well as animal suffering. Furthermore, species selection plays an important role since their level of development, size, trainability and cooperation with the human handlers will impact evaluation methods.

9.1. Non-invasive diagnostic imaging methods

In musculoskeletal research the benchmark for assessing recovery is usually composed of diagnostic imaging modalities, such as plain radiographs, computed tomography (CT), magnetic resonance imagine (MRI) and magnetic resonance (MR) spectroscopy, scintigraphy, ultrasound and fluoroscopy. All of these methods have the advantage that they allow *non-invasive* evaluation of the target of assessment over time. For quality and reasons of restraints animals normally have to undergo general anesthesia for these procedures, except for possibly radiographs, standing MRI in horses[38] and modern fluoroscopy, where ambulation with weight bearing can be visualized.[39] In these situations sedation may be required depending on individual animal behavior. It is imperative that SOPs are used to standardize data collection so that datasets that can be compared between and within animals over time.

Although diagnostic imaging offers tremendous possibilities, caution is required for interpretation of results, especially if biomaterials, such as calcium phosphate cements or metallic implants are used. The latter cause artifacts in the immediate environment of CT images and complicate the interpretation of the material to bone-contact-interface (BIC).[40] The use of high-field MRI can also be problematic with metal implants due to concerns about implant migration and possible local heating effects. Materials consisting of calcium aggregates also deliver dubious results in CT scans, since the equipment cannot distinguish between hydroxyapatite of natural bone or calcium scaffolds /matrices. In addition, results are also dependent on the general threshold of calcium detection set for the scans. Therefore, CT scans used for detection of new bone formation or material resorption should be combined with histology of non-decalcified bone samples.

MRI has the advantage of showing soft tissue structures and can be applied for almost all aspects of musculoskeletal research. However, one has to keep in mind that the power of the MRI equipment may determine the successful detection of treatment differences. For example, a 1.5 Tesla MRI was not reliable for interpretation of changes of hyaline cartilage,[41] while 3 or 7 Tesla equipment was more suitable. Spectroscopy can also deliver valuable information about type of tissue molecules (proteoglycan, fat, etc.).[42] Ultrasound is feasible for screening tendons for signs of degenerations, ruptures, or fluid accumulation within soft tissue and is routinely used in horses.[43]

9.2. *Minimally-invasive Assessment and Sampling*

Arthroscopy is the most prominent method for “second-look” evaluations in joints following imaging procedures above. The procedure gives direct visualization under good illumination and high magnification, while also allowing for biopsies. Research projects examining cartilage resurfacing can benefit greatly from arthroscopy.[44] If performed correctly, arthroscopy results in minimal damage and does not severely disturb the overall course of healing/degeneration of hyaline cartilage or other associated joint structures.

Serial biopsies are a good method to assess functional outcomes following surgery and are easily performed in most cases as long as the biopsy material is removed in a manner (and from a location) that does not negatively influence healing and remodeling of the residual tissue. The same is true for sampling synovial fluid over time with repeated punctures. However, it has to be kept in mind, that even with these minimally invasive methods perioperative inflammation is produced within the joint structure and therefore, these procedures should be temporally spread apart, such that results are not artefactually influenced by the previous procedures.

9.3. *Biomechanical Methods*

When considering biomechanical testing, it has to be determined whether tests are to be performed *in vivo*, potentially with multiple time points, or whether tests are conducted post-mortem to provide only a single time point. This will likely depend on what data you are trying to capture and the equipment available to the investigator. By combining multiple methods a global picture of the structural and material properties can be obtained.

Kinetic analysis of ground reaction forces, whether by traditional force plate or the more recently developed pressure-sensitive walkways,[45] provides for objective functional assessment of overall limb use. Combining kinetic outputs with kinematic data, obtained from motion capture systems, allows for real-time monitoring of changes in both limb use (overall loading) and limb function (changes in range of joint motion).[46] These combined data can then be imported into commercial or open source simulation software such as OpenSim to allow for the calculation of joint loads and the development of mathematical models of joint function.[47] Muscle activation during activity can also be determined by means of electromyography (EMG) using either invasive (needle electrodes) or surface recording.

Advances in microsensor and telemetry technology are now making it possible to obtain real-time output from tissues or implants in vivo. For example, strain gauges implanted in/on tissues or around joints can be used to record functional loads in vivo.[48] Although

expensive to deploy, these implantable devices allow for real time collection of serial data from individual animals, allowing for reductions in overall animal numbers.

Measuring indentation is a method to assess mechanical properties of hyaline cartilage and can be performed *in vivo* or *ex vivo*.[49] Standardization of the method may be tricky especially in vivo, since setting the instruments at the correct 90° angle may not always be easy. More recently, reference point indentation has been shown to be a robust, nondestructive method for obtaining quantitative data on the mechanical properties of whole bones.[50]

Ex-vivo methods of biomechanical testing incorporate mostly measuring tensional, compressive and/or shear forces or fatigue of (healed) tissue structures in customized settings. The classic example would be a materials testing machine that can test the mechanical properties of whole limbs, or individual elements such as bone or tendons. If working with biomaterials, removal torque or push-out tests are often used to evaluate osseointegration.[51]

9.4. Histology

Histology is a valuable tool for assessing functional outcomes after surgical or medical treatment. Structural as well as cellular changes can be observed in detail, although one has to be aware that it is a two dimensional method that often suffers from limitations in terms of sampling frequency, making its general applicability to the tissue as a whole more limited. However, these limitations can be offset to some extent through the use of stereology, as well as by combining serial sections with 3-dimensional computed tomography and/or MRI imaging to provide the third dimension.

The type of histology that is performed will depend on tissue type and whether implants are left *in situ* (e.g. metallic implants). If metallic implants are to be sectioned in situ, non-decalcified tissue samples are embedded in a hard epoxy or acrylic resin such as polymethylmethacrylate, then sectioned using a bone saw with a diamond band saw.[52] The cut sections are then ground and polished to final thickness (usually 100-150 µm)

and surface stained with either toluidine blue or Giemsa. Toluidine blue is a convenient histochemical staining to assess new bone and/or cartilage formation.

If thin bone sections are required for studying tissue responses at a cellular level, any metal implant will need to be removed. The tissue is embedded in acrylic resin as above, then sectioned on a rotary microtome or annular saw. Thin sections allow for the use of a broader variety of stains, including toluidine blue, Movat pentrachrome, von Kossa/McNeal, hematoxylin eosin, etc. Staining protocols depend on the specific question that is being asked. Although possible, immunohistology with plastic sections is problematic and is most reliable in bone samples from small rodents and there, preferably bone marrow and not cortical bone. Special resins are available, which are mostly too soft for sheep bone and make it almost impossible to get reliable and repeatable results, especially if cortical bone is involved.

If there are no implants in the tissue, or if removal of the implant is feasible, decalcification and processing into paraffin is the preferred technique for bone sections. Decalcification is relatively straightforward requiring hours to days depending on the bone thickness and density. Immunohistochemistry is frequently unreliable because of the fixation and decalcification process, thus having reliable validated antibodies is necessary.

Frozen sectioning is technically challenging for bone but feasible for cartilage and soft tissues such as muscle, ligament, tendon, or fibrous tissue. Tendon tissue from larger animals like the horse may be too dense to get good and reliable frozen sections, thus paraffin sections or even plastic sections may be more suitable. For cartilage alone, cut off from the calcified zone, frozen sections and paraffin sections are commonly used and suitable for most assessments. Also, identification of fat in tissues is best performed on frozen sections because xylene leaches out the fat droplets during processing. Other techniques for identification of fat have proven successful if frozen tissues cannot be used.

Immunohistochemical analysis is highly dependent on the antibody. Tissue preparation can greatly influence whether an antibody is going to be successful, and simply working in western blot analysis is no guarantee that it will also work in tissue sections. Frozen sections are often the most reliable for antigen detection, followed by tissues preserved in a short (less than 24 hours) exposure to paraformaldehyde, formaldehyde, long exposure (greater than a day) in either paraformaldehyde or formaldehyde, and finally the least successful are decalcified tissues. Antigen retrieval techniques are also available to increase the likelihood of immunohistochemical staining success.

Electron microscopy uses beams with accelerated electrons to study the ultrastructure of specimens. Transmission (TEM), scanning (SEM), reflection (REM) and scanning transmission electron microscope (STEM) are provide high spatial resolution. Confocal light microscope sometimes overlaps with electron microscopy and for each particular research question, pilot studies may be needed to determine the most appropriate method of analysis.

9.5. Molecular and biochemical methods

Modern research in the musculoskeletal area also involved methods of molecular biology in which DNA, RNA or specific proteins are quantified. The specifics of these techniques are well beyond the scope of this review and will not be presented in detail, beyond reminding the reader that the isolation of intact, high quality RNA from connective tissues can be challenging and requires efficient and rapid processing of tissues following collection from the animal. In addition, the low density of cells in soft tissues such as tendons and ligaments requires extensive processing for retrieval of the DNA and RNA in the vast collagen milieu.

10. Veterinary Clinical Trials as a Bridge between Preclinical Laboratory Animal Studies and Human Clinical Trials

There are many opportunities in the veterinary clinical realm to utilize patients to 1) demonstrate efficacy of an orthopaedic surgical procedure or 2) investigate efficacy of a non-surgical treatment modality. Utilizing veterinary patients can potentially bridge the gap between studies in preclinical laboratory animal models and human clinical trials,

leading to proof of efficacy and benefits for the veterinary population, as well as providing a "pathway" for drugs and procedures to be further studied in humans.[53]

In the veterinary clinical population there are many patients that provide excellent examples of a naturally occurring disease where intervention may be very similar to those in human patients, including osteoarthritis (OA)[54] and bone cancer.[55] A challenge in this regard is that even within a disease category such as OA, there may be significant differences between species in terms of disease onset (acute or chronic), progression (weeks verses months), onset of clinical symptoms versus when the disease began, etc. If the outcomes of the disease is not understood in depth, this could lead to misinterpretations of a disease treatment and false positive or negative results that will not translate successfully into human clinical trials. It therefore is important for the clinician scientist evaluating new treatments to be familiar with the current standard of care for this condition in human and/or veterinary patients.

Another opportunity in veterinary patients is that issues such as placebo effects are much more rare due to the nature of the patient however the evaluator (clinician) can certainly be biased. The evaluators of the treatment should remain blinded to the therapy so as to prevent undue bias. Just like in human clinical trials, the use of randomized blinded (it is double-blinded in people) clinical studies is warranted to make the results more robust. Cook et al published an article with proposed definitions and criteria for reporting time from outcome and complications in veterinary clinical studies and strict definitions are described to aid veterinary clinical scientists in using similar terminology to human clinical trials.[56]

When outcome parameters are described in veterinary clinical patients, variability can be introduced due to pre-existing issues with outcome assessments. For example a horse is subjectively scored based on a typical 0-5 lameness scale and flexion tests but dogs are graded on criteria such as activity, mood, playfulness etc., which also is very different from how pain is assessed in humans. While more objective analyses are being developed for both the horse and dog in terms of gyroscopic lameness detectors (Lameness Locator®)[57] and force plate analyses, these outcome parameters are very

different to the pain scales utilized in humans, making it very important to select outcome measures appropriately. The use of validated survey instruments for assessing pain and functional impairment shows considerable promise in this regard.[58, 59]

Further challenges that are similar to both veterinary and human clinical trials are recruiting patients and getting a population that is similar in disease state. Further, once a treatment or procedure is pursued in a veterinary population, the patients are no longer in a "controlled" environment. Study PI's rely on owners to interpret recommendations of how to care for the patients but compliance is not always consistent. A more "hybrid" model has been followed recently in which horses with OA were treated and kept within a center for the entire period of assessment. This accomplished several objectives that allowed maximum consistency; a controlled environment in which patients had consistent care, access to equipment such as force plate analysis and a lameness locator, and the ability to closely monitor responses that may otherwise have been missed.[60] While this approach may initially seem more expensive, the reduction in variability in data collection, combined with the increased compliance of study animals means that the overall cost for these hybrid studies may be lower than with an outpatient field trial (Bertone A, Personal communication).

An opportunity that exists in veterinary patients is that the majority of patients are not covered by insurance policies therefore owners are often motivated to enter their animals onto a clinical trial especially if there is a monetary incentive (to cover some or all of their medical expenses). Conversely, a challenge of these studies is that owners may be unwilling to participate if there is a possibility that their animal may receive the placebo control treatment. To motivate owners to enter their animals onto a clinical study, a crossover design may be an important incentive needed to partake in a trial where all animals receive treatment eventually (either at the initiation or following treatment with the control).[61] As in all clinical trials whether in people or in veterinary patients the importance of accurate power analyses cannot be stressed enough. If these studies are underpowered, the value of the conclusions are meaningless and more importantly, a potentially effective treatment or surgical procedure is assumed falsely effective (dangerous for the patients) or falsely ineffective resulting in a missed

opportunity to bring a valuable therapy to fruition. Biostatisticians that are well versed and familiar with biological studies should always be included as valuable team members of clinical trials to ensure proper analyses are performed and that there is no bias associated with the results.

11. Summary and Conclusions

Our ability to continue to use laboratory animals in biomedical research is under more intense scrutiny than ever before. The balance has shifted so that the burden of evidence now lies squarely with the researcher, who must justify the clinical/translational relevance of his/her research and demonstrate that the experimental methods do not cause undue pain, distress or suffering to the study animals. Recent initiatives within the ORS and other allied organizations are intended to enhance the training opportunities available to investigators at all career stages, to provide a network of researchers capable of mentoring young investigators, and to offer timely reviews in the form of white papers on best practices in animal model selection, experimental design/conduct, and study reporting. It is our hope that through these initiatives, we will be able to demonstrate to the public that orthopaedic researchers understand the absolute need to consider and then apply the fundamental principles of the 3Rs when undertaking in-vivo research studies in animals.

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