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Review Article

Mouse fracture models: a primer

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Abstract: Mouse fracture models have become increasingly versatile tools for the study of how fractures heal. Mouse genes can be specifically modified using transgenic technology, thereby providing the opportunity to study the roles of individual genes during the fracture healing process. The mouse rib fracture model is a reliable model for use in studies of gene expression during fracture healing, which do not involve fixation or biomechanical testing. The versatility of the mouse femur fracture model is based on the fact that femoral anatomy makes femur fractures more easily fixed and reproducible than other mouse fracture models. To investigate the mechanism of normal fracture healing, a mouse femur or tibia is usually fractured using a 3-point bending device and is then closely fixed with special devices. To investigate issues involving delayed healing and non-union formation, the mouse femur is often osteotomized and then openly fixed using different devices as needed. The newest generation of 3-point bending devices allow for generation of reproducible transverse femur fractures in mice of different ages and sizes. Methods to assess fracture healing range from conventional radiological, histological and biomechanical analyses to MRI, micro-CT, radioisotope imaging, and specific molecular and genetic assays. Live gait analysis can also be performed if needed. Overall, current mouse fracture models provide a large array of validated and standardized protocols to analyze physiological, biomechanical, histological, molecular, and genetic aspects of normal and pathological fracture healing. The present review summarizes some of the most common techniques and their applications.

Keywords: Animal model, mouse fracture model, bone repair, mice, fracture stabilization

Introduction

Multiple animal fracture models are available for the study of bone healing [1-7]. Traditionally, large-animal models have been preferred, including dog, rabbit, goat and sheep models [2, 3, 5, 8, 9]. Large-animal bone remodeling closely mimics that in humans because the bones of both exhibit Haversian systems [10-12]. In contrast to larger animals, mouse bone remodeling occurs via resorption cavities [13, 14]. Although large-animal bones can be used for implant stabilization [2, 3, 8, 11], a major disadvantage of their use is the high cost associated with maintaining their housing during the long healing period, which may exceed what can be realistically covered in the current environment of increasingly competitive funding. Therefore, with the development of modern molecular and genetic techniques, smaller animal models have become increasingly popular and versatile [13-16]. A large variety of custom and genetically altered mouse models, as well as a broad spectrum of mouse antibodies, have been developed, and the wide availability of these tools relative to those available for other species has fostered increasing interest in mouse models for various orthopedic applications [13-15].

Developing a standardized fracture model in mice remains a major challenge. This model should exhibit consistently reproducible features, including the type, site, and degree of fracture displacement and soft tissue injury. Surgical treatments and the rigidity of fixation constructs should also be reproducible from specimen to specimen. Reproducible features of fracture displacement and soft tissue injury can be achieved by using a standardized bending or alternative mechanical fracture device. The small size of mouse bones makes fracture fixation a challenging task; thus, long bones, such as the femur and tibia, are primarily used

to study fracture healing involving fixation or biomechanical assays [17, 18]. Various implants have been used in mouse fracture models [4, 13-15, 19-25]. Different implants and surgical techniques result in different biomechanical fracture-healing environments, which can significantly influence bone healing processes and outcomes [10, 13-15, 19-22, 26-34]. The present review describes the advantages and disadvantages of standardized bending and fracture devices and of different surgical techniques and fixation devices.

Advantages of the mouse fracture model

Compared with large-animal models, mouse models possess distinct advantages. Laboratory mice are, in general, genetically well-defined [35-39]. Genetically manipulated mice allow for the study of distinct molecular mechanisms involved in the bone healing process [35, 36]. Furthermore, there is a large array of commercially available mouse monoclonal antibodies, providing a large number of markers and tools for studying specific in-vivo molecular targets. This variety of available antibodies allows researchers to address the specific contributions of these various molecular targets to the process of bone remodeling [35]. These types of experiments are not often performed in larger animals due to a lack of genetically modified model species and the limited availability of monoclonal antibodies [35].

Furthermore, financial considerations are increasingly relevant as research budgets continue to shrink. For example, a 20-g mouse is significantly more economical to acquire, house, feed, and dispose of than a 50-kg sheep. A large number of mice can be kept in a small space, whereas large animals often have to be housed away from research centers and transported to research centers each time assessments are performed. Moreover, mouse breeding cycles are substantially shorter than those of larger animals; thus, a sufficient number of mice with specific genetic profiles can be obtained to form large study groups in a reasonable amount of time.

Mouse selection

Differences in age, sex and mouse strains influence fracture healing biology [40]. For example, when compared with healing in DBA/2 and C3H inbred strains, fractures in C57BL/6 mice heal more rapidly [40], showing that genetic variability significantly contributes to the process of bone remodeling and healing.

Sex also influences fracture healing. Female mice exhibit a reduced maximum torque at failure when compared with males [41]. In addition, the bone marrow in the femora and tibiae of female mice contains fewer mesenchymal stem cells (MSCs) [42].

Age affects fracture healing as well. Aged mouse osteoblasts exhibit a decreased response to osteogenic stimuli and a delay in chondrocyte differentiation and maturation, which results in delayed endochondral ossification [43]. Age is also associated with the diminished expression of factors that regulate angiogenesis, thereby affecting the process of vascularization during fracture healing. Aged MSCs also show a decreased rate of tissue repair and regeneration [44]. Thus, age is a relevant factor that should be considered in fracture healing studies. Mice are sexually mature at 6 to 8 weeks of age and undergo physeal fusion at that time; therefore, mice of this age are often selected for fracture studies because their bones are no longer growing in size.

A consistent weight between individuals is also a desirable characteristic in animal studies of bone fixation because bone size closely correlates with weight. Additionally, the surgical fixation of bones of different sizes should be avoided in any study. The use of mice weighing over 20 g is practical because mice of this size often have femora that are 2 to 2.5 mm in diameter. For animal studies of bone fixation, close attention should be paid to acquiring mice that are both age- and weight-matched.

Fracture healing models in mice

Rib fracture model

The rib fracture model remains a useful tool for studies that do not involve fixation or biomechanical testing [45]. Under inhaled anesthesia, the eighth rib on the right side can be exposed and cut vertically along the long axis of the rib using scissors [45]. This model has been successfully used to examine gene expression during fracture healing [46-49].

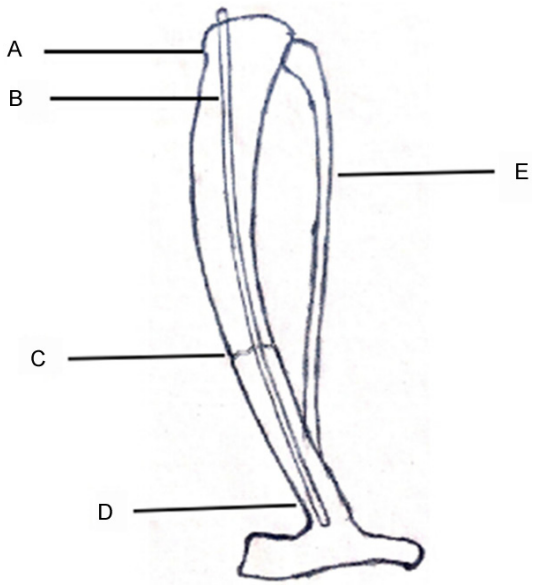

Figure 1. Tibia fracture model: A. Proximal portion of the tibia; B. Stainless-steel pin; C. Fracture line; D. Distal portion of the tibia; E. Fibula.

Tibia fracture model

The closed tibia fracture model is well-described and involves the use of stainless steel fixation pins [50] (**Figure 1**). In one study, Bonnarens fixed the tibia using a 0.2-mm stainless steel pin prior to fracturing the bone with a 3-point bending device [51], which resulted in a reproducible transverse or slightly oblique fracture pattern. Special attention should be given when using the tibia fracture model. For example, it is necessary to control whether the fibula is broken, as the status of the fibula will affect the overall stability of tibial fixation and can influence the mechanical healing environment [52]. The tibia fracture method was adapted from the closed femur fracture model in rats [51]. Mechanical testing procedures can be conducted using the tibial fracture model, and this model has been successfully used to examine gene expression during healing [53-56].

The main technical advantages of the tibia fracture model include its reduced surgical invasiveness, low implant weight, and low cost. The primary disadvantages of this model are the lack of both axial and rotational stability when using a pin, the high risk of knee dislocation, and the potential for intramedullary cavity damage. When using the tibia fracture model in mice, the shape of the implanted stainless steel pin should be modified to match the curved longitudinal axis of the tibia to facilitate its introduction into the medullary cavity.

Femur fracture model in mice

The mouse tibia allows easier intramedullary access than the femur; however, the curved major axis of the tibia complicates biomechanical testing. The minimal amount of local soft tissue surrounding the bone may also result in healing and soft-tissue envelope complications [20-22, 57]. Furthermore, the proximity of the fibula to the tibia may influence the healing rate if the fibula is accidentally fractured, which can occur at rates of up to 30% [20-22, 57]. In contrast, the femur is a tubular bone that is more thickly covered in by muscle, and the diameter of the femur is relatively consistent and large compared with that of the tibia, which facilitates the use of larger implants, such as screws for plates, as well as internal and external fixators [20-22, 57]. The following techniques are presently available for stabilizing long-bone fractures and are predominantly used in femur fracture models in mice.

Intramedullary pin

The closed femur fracture model in mice using intramedullary pin fixation is based on the well-established closed femur fracture model in rats [51]. Prior to fracturing the femur using a 3-point bending device, a 0.2-mm stainless-steel pin is inserted into the medullary cavity of the femur [51] to maintain axial alignment during the fracture and avoid large displacements. Compared with its use in the tibia, this method of pin fixation prior to femur fracture in mice is not stable against longitudinal and rotational deformations.

This method facilitates control of the fracture site and results in a standardized fracture healing environment. This model can be used to create a standard fracture, and the intramedullary pin can be removed to study other aspects and effects of fracture healing.

Locking nail

In the locking nail system described by Holstein, a modified 24-gauge injection needle serves as the locking nail, and a 0.1-mm-diameter tung-

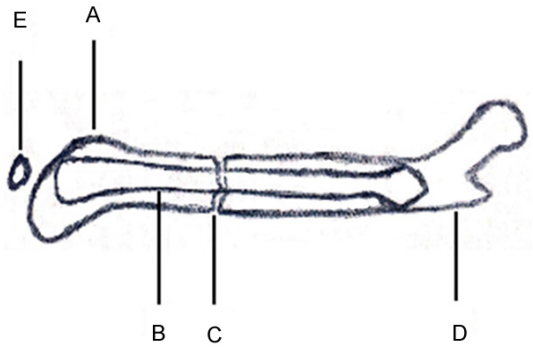

Figure 2. Femur fracture model (Locking nail): A. Distal portion of the femur; B. Locking nail; C. Fracture line; D. Proximal portion of the femur; E. Patella.

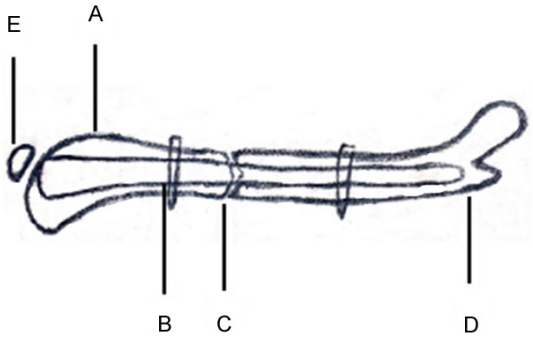

Figure 3. Femur fracture model (Interlocking nail): A. Distal portion of the femur; B. Interlocking nail; C. Fracture line; D. Proximal portion of the femur; E. Patella.

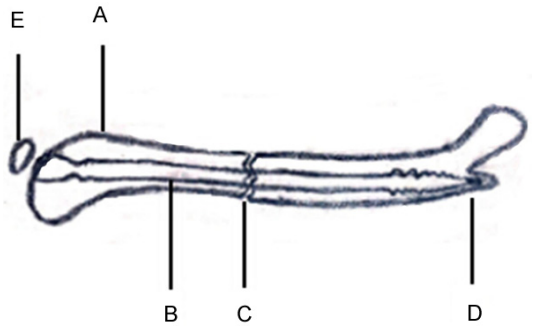

Figure 4. Femur fracture model (Intramedullary compression screw): A. Distal portion of the femur; B. Intramedullary compression screw; C. Fracture line; D. Proximal portion of the femur; E. Patella.

sten guide wire is used for its insertion [58] (**Figure 2**). During the surgical procedure, a 0.1-mm-diameter tungsten guide wire is inserted into a hole drilled into the intramedullary canal at the intracondylar notch using a 0.5-mm-diameter trephine. A closed diaphyseal fracture is then produced using a 3-point bending device, and the modified 24-gauge injection needle (a spearhead-configured needle) is introduced over the guide wire [58]. After the guide wire is removed, the distal end of the needle is flattened at a right angle to the proximal spearhead and then pressed into the intracondylar notch. Flattening the proximal and distal ends of the needle assures rotational stability of the femur fracture. Although this technique offers higher stability compared with that of the simple pin fixation method for the closed femur fracture model in mice, the locking nail system is not a rigid fixation technique and is suitable only when a relative stability model is intended. Similar to the simple pin fixation method, this model maintains the advantages of minimal invasiveness, surgical simplicity, low implant weight, and low cost. The main disadvantage remains the potential for damage to the intramedullary cavity.

This fracture model provides a simulation of a clinical trauma setting, and minor surgery is required to stabilize the fracture and provide rotational stability. The technique is appropriate for studying fracture healing.

Interlocking nail

To achieve a more rigid fixation of femur fractures in mice, an intramedullary nail was designed by Garcia using micro-CT data (31). This device can be locked proximally and distally by two pins (0.3 mm in diameter) using a specially designed targeting arm in a manner analogous to intramedullary fixation in humans [57] (**Figure 3**). This system involves a 0.8-mm-diameter intramedullary nail, which requires open fracture stabilization. In this model, the femur fracture is performed using an open osteotomy technique. Because the size of the gap created during the osteotomy can be controlled by the operator, this model is ideally suited to study normal fracture healing, delayed healing, and non-union formation. However, the cost of the device is high, and the open technique and associated soft tissue disruption may not be desirable under some conditions. The primary advantage of this technique is the high degree of axial and rotational stability it achieves. The major disadvantage is that it involves a complex, invasive surgical procedure, which includes collateral damage to the intramedullary cavity.

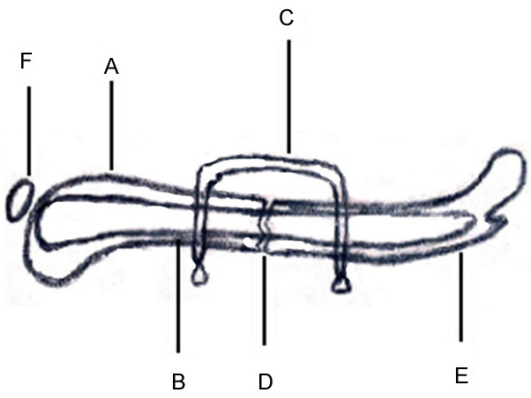

Figure 5. Femur fracture model (Pin-clip device): A. Distal portion of the femur; B. Pin; C. Clip; D. Fracture line; E. Proximal portion of the femur; F. Patella.

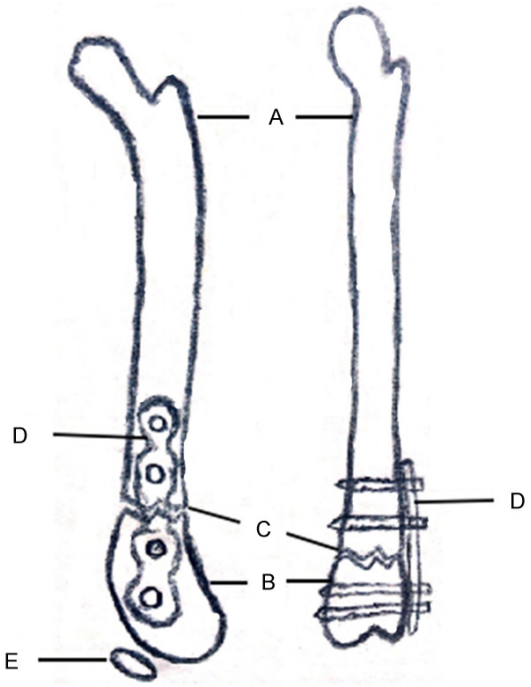

Figure 6. Femur fracture model (Locking plate): A. Proximal portion of the femur; B. Distal portion of the femur; C. Fracture line; D. Locking plate; E. Patella.

Due to the associated axial and rotational stability, the interlocking nail method can be used in a wide range of research on bone healing in mice.

Intramedullary compression screw

To achieve rotational and axial stability after the fixation of a closed femoral fracture in mice, an intramedullary compression screw (length: 18 mm and diameter: 0.5 mm) can be used, thereby establishing a closed, stable fracture model without traumatic surgery [21, 59] (**Figure 4**). This technique introduces a guide wire prior to fracturing the bone and before the insertion of the cannulated screw implant. The screw can rotationally and axially stabilize the fracture by compressing the femur at the site of the fracture. This method is considered a rigid fixation technique. This model maintains the advantages of a less invasive, simple surgical technique and a low implant weight. The associated disadvantages include a higher implant cost and the potential for damage to the intramedullary cavity. This model may be suited for studying the molecular mechanisms of normal fracture healing and is less useful for non-union studies.

Fractures in this model are fixed using a rigid fixation technique. Therefore, this method can be used to study the effects of post-operative exercise and to identify and develop effective post-operative exercise regimens.

Pin-clip device

To develop a reliable non-union model in mice, the pin-clip device was introduced to simultaneously achieve rotational and axial stabilization using an intramedullary pin [60] (**Figure 5**). The pin-clip device is fixed to the femur fracture by exposing the femur using an open surgical technique. This method allows for the creation of fractures with different gap sizes and is suitable for studying the mechanisms of normal fracture healing, delayed healing, and non-union formation. The advantages of this model include its high axial and rotational stability, low implant weight, and low cost. The major disadvantages are the need for an open surgical procedure and damage to the intramedullary cavity.

This device provides rotational stability and guarantees a standardized osteotomy, which allows for the study of defect healing. Therefore, this technique may serve as an ideal alternative to external fixation techniques.

Locking plate

Whereas the use of intramedullary fixation has predominated in the literature, locking plates with locking screws have been used for diaphyseal or metaphyseal open fracture models in

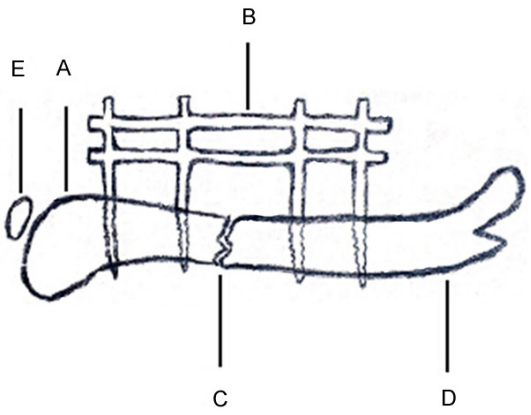

Figure 7. Femur fracture model (External fixator): A. Distal portion of the femur; B. External fixator; C. Fracture line; D. Proximal portion of the femur; E. Patella.

mice as a system of extramedullary fixation [20-22] (**Figure 6**). This system is intended for attenuating periosteal damage by minimizing implant-bone contact. The locking plate is fixed to the bone using 4 interlocking screws, which achieve stable, rigid fracture fixation via open surgery. The plate method also allows for the study of normal fracture healing, delayed healing, and non-union formation via the stabilization of different gap sizes without traumatizing the intramedullary canal or its vascular system.

The locking plate method allows for the study of metaphyseal bone healing in mice under mechanically defined and standardized conditions.

External fixator

The external fixator technique in mice is analogous in design to those used in clinical practice for humans (**Figure 7**). The external fixator consists of a fixator block and four mini-Schanz screws (AO Development Institute) [15, 21]. The four screws, which are drilled into the proximal and distal bone fragments, are used to connect the block to the bone. Although the fixator does not affect the fracture zone, the application of the screws can be traumatizing to the surrounding soft tissue. After the fixator has been attached to the intact femur, a fracture is created by drilling holes in the mid-shaft region of the femur and manually bending the bone. The application of the external fixator allows for the study of normal fracture healing, delayed healing, and non-union formation via the stabilization of fractures with different gap sizes [61]. The main disadvantages are the relatively high weight of the fixator, high cost of the implant, and the potential for the bulky external fixator to restrict physiological activity and the gait of the animals, which can lead to self-injury by the animals and can cause subsequent infections.

The femur fracture model stabilized by external fixation more closely mimics techniques used in clinical cases and is similar to the open femur fracture cases treated with external fixators.

3-point bending device to create closed fractures in mice

The fracture device most widely utilized in mouse models is the 3-point bending, gravity-driven fracture device [21], which was first described for use in the rat tibia fracture model by Bonnarens and Einhorn [51]. The simple gravity-driven, 3-point bending design is easy to construct, operate, and maintain. However, three potential disadvantages are of note. The femur's location and small size in mice make the proper positioning of the bone on the device difficult. Moreover, the reset spring of the device may experience metal fatigue with use, which can result in inconsistent fractures over time. Additionally, smaller transgenic mice present a challenge to producing consistent fractures [22].

A newer generation gravity-driven fracture device addresses these issues [62] by offering improved femur positioning, consistent impact velocity, and adjustable kinetic energy inputs. These new devices conform to the demands of the anatomic structure of the mouse leg, and the return spring is eliminated, which results in a more consistent impact velocity and optimizes the device's performance. Being able to control the kinetic energy input allows for reproducible transverse fractures via adjustments of the impact mass and velocity. With these improvements, an added advantage is that mouse weight becomes an insignificant determinant of the fracture type in a closed fracture model.

Anesthesia in fracture healing models

Injectable (intraperitoneal) anesthetics are primarily applied in surgical procedures for the

majority of the published research on mouse tibia or femur fracture models but not in those on rib fracture models [19-22, 57]. Because animals often have to be manipulated and can undergo various positional changes during fracture induction and repair, intraperitoneal anesthetics are more practical. Inhaled anesthesia, which is commonly used in research with rib fracture models, requires the use of nose-cone ventilation during surgery and obstructs the mouse position during surgical biomechanical experiments. The most common injectable anesthetics are 2 mg/kg xylazine and 75 mg/kg ketamine, which are low in cost, easy to administer, and pose no health risks to the investigator [19-22, 57].

Fracture healing assays

Image analysis

In most cases, to study radiological changes of the fracture healing process in mice, specimens must be euthanized at different time points after fracture induction [19-22, 57]. Usually, high-resolution radiography and 2-dimensional or 3-dimensional microcomputed tomography (micro-CT) are used to assess the fracture healing process in mice [19-22, 57]. Conventional x-ray techniques are able to differentiate the size and radiological density of the fracture callus [19-22, 57]. Micro-CT scans can reveal detailed information about tissue mineral density, total callus volume, and the bone volume fraction of the callus [19-22, 57].

Noninvasive real-time imaging techniques have been introduced in the past few years to evaluate gene expression, protein degradation, cell migration, and cell death during the bone repair process in living animals. Techniques involving bioluminescence, near infrared fluorescence, and nuclear and magnetic resonance imaging are all highly useful [63-65]. Although micro-CT scans can also be applied in vivo, ex vivo micro-CT scans provide a significantly higher resolution than in vivo scans [66]. The vasculature of the callus can also be visualized and quantitatively assessed using ex vivo 3-dimensional micro-CT. Moreover, ex vivo 3-dimensional micro-CT combined with the use of a contrast agent can resolve the vasculature of the callus [66].

Histomorphological analysis of fracture healing

A histological technique has been developed to assess and analyze the remodeling process of the callus. This technique is sufficient for distinguishing osteoblasts and osteoclasts, the anabolic and catabolic rates of these cells, and the structural features of the remodeled callus [19-22, 57].

In general, after the healed specimens are resected and the implants removed, the bones are fixed, stained, and analyzed histomorphometrically following the commonly applied guidelines of the American Society of Bone and Mineral Research (ASBMR) [67].

Given the 3-dimensional structure of the bony callus, it is necessary to define representative, standardized parameters for a reproducible calculation of the size and tissue composition of the callus [19-22, 57]. These include (1) total callus area/bone diameter at the fracture gap, (2) bone callus area/total callus area, (3) cartilaginous callus area/total callus area, and (4) fibrous callus area/total callus area [19-22, 57].

Biomechanical analysis

Nondestructive 3-point bending, destructive 4-point bending, and torsion or axial testing have all been used to study the biomechanical properties of bone repair in mouse tibia and femur fracture models [19-22, 57]. In contrast, the anatomical structure of the rib is irregular to the point that it is not amenable to biomechanical studies.

A nondestructive 3-point bending test has been used to measure callus stiffness in a femur fracture model in mice using different fixation techniques [20]. During nondestructive tests, loading is most often stopped when the load-displacement curve deviates >1% from linearity, and the conformation of a nondestructive loading protocol is performed macroscopically and histologically. The bending stiffness (N/mm) can be calculated from the linear elastic portion of a load-displacement diagram [20].

A 4-point destructive bending test has been used to measure the ultimate bending stiffness (N/mm) and bending load (N) of the tibia in a

mouse tibia fracture model [17, 68]. The ultimate bending load is defined as the maximum load at failure, which is determined directly from load-deformation curves. The ultimate bending stiffness can be measured based on the slope of the linear elastic section of the curves.

Torsion or axial testing have been applied to a femur fracture model with intramedullary fixation to determine fixation effectiveness [22, 58].

Overall, the smaller size of mouse bones represents a great challenge for biomechanical testing relative to tests involving larger specimens and thus requires highly sensitive testing devices. In general, the results of any biomechanical analysis of healing bone are expressed as a percentage of the results from the contralateral intact bone to account for the individual differences of the animals.

Immunohistochemical analysis

In addition to histomorphometric studies, immunohistochemical analyses allow for the in situ spatial detection of different proteins, such as cytokines and cell markers, within the fracture callus [50, 68, 69].

The results of immunohistochemical assessments can be supported by semiquantitative protein analyses using biochemical methods such as Western blotting and enzyme-linked immunosorbent assay techniques [70]. In situ hybridization studies provide further information on the corresponding messenger RNA expression in the different cell types [68]. Additionally, the assessment of in situ messenger RNA expression can be supported by semiquantitative techniques such as Northern blotting and reverse transcription-polymerase chain reaction (RT-PCR) analyses [68]. Furthermore, cells of the fracture callus can be harvested for additional cell culture studies.

In vivo gait analysis

Gait analysis is a powerful technique that can be used to evaluate patterns of animal motion after surgery [71, 72]. A novel technique for gait analysis has been introduced in the mouse femur fracture model with intramedullary pin fixation to test for changes in movement patterns after fracture and fixation [72]. Dynamic gait analyses provide continuous data on the tibiofemoral angle via digital video-radiography. In this technique, the range and maximum value of the tibiofemoral angle is the crucial parameter [72]. Fracture fixation resulting in a significantly reduced range and peak value of the tibiofemoral angle compared with those of the non-fractured controls implies a significantly reduced stride length. Significant alterations in the gait of mice have been observed when comparing different fracture stabilization techniques.

Conclusion

A variety of different mouse fracture models are available for studying the cellular and molecular mechanisms of fracture healing. Open or closed models used with or without different fixation techniques to investigate normal fracture healing, delayed healing or non-union formation are accessible to most investigators.

Comparative analyses should be conducted using mice of the same age, weight, sex and strain to minimize variability among and within the study groups. To produce a closed femur fracture model in mice, new 3-point bending devices are available that allow for generation of highly reproducible transverse femur fracture patterns. In studies using these new devices, mouse weight does not need to be considered as an influential factor.

Current mouse femur fracture models provide standardized methods for researchers to analyze molecular and genetic aspects of normal fracture healing, delayed healing and non-union formation.

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Disclosure of conflict of interest

None.

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