Journal of Orthopaedic Translation (2015) 3, 95—104

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: http://ees.elsevier.com/jot

REVIEW ARTICLE

Bone defect animal models for testing efficacy of bone substitute biomaterials

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Received 11 March 2015; received in revised form 21 May 2015; accepted 21 May 2015
Available online 16 June 2015

KEYWORDS
animal models;
bone defect;
bone regeneration;
bone substitutes

Summary Large bone defects are serious complications that are most commonly caused by extensive trauma, tumour, infection, or congenital musculoskeletal disorders. If nonunion occurs, implantation for repairing bone defects with biomaterials developed as a defect filler, which can promote bone regeneration, is essential. In order to evaluate biomaterials to be developed as bone substitutes for bone defect repair, it is essential to establish clinically relevant *in vitro* and *in vivo* testing models for investigating their biocompatibility, mechanical properties, degradation, and interactional with culture medium or host tissues. The results of the *in vitro* experiment contribute significantly to the evaluation of direct cell response to the substitute biomaterial, and the *in vivo* tests constitute a step midway between *in vitro* tests and human clinical trials. Therefore, it is essential to develop or adopt a suitable *in vivo* bone defect animal model for testing bone substitutes for defect repair. This review aimed at introducing and discussing the most available and commonly used bone defect animal models for testing specific substitute biomaterials. Additionally, we reviewed surgical protocols for establishing relevant preclinical bone defect models with various animal species and the evaluation methodologies of the bone regeneration process after the implantation of bone substitute biomaterials. This review provides an important reference for preclinical studies in translational orthopaedics.

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http://dx.doi.org/10.1016/j.jot.2015.05.002

Introduction

Bone defect healing is a process of reconstruction of the bone tissue, which generally undergoes a multidimensional procedure with an overlapping timeline [1]. The vast majority of bone defects can heal spontaneously under suitable physiological environmental conditions due to the regeneration ability of bone. However, the healing process of bone defect is time consuming, and new bone generation takes place slowly because of decreased blood supply to the fracture site and insufficiency of calcium and phosphorus to strengthen and harden new bone. In addition, large defects, also known as critical bone defects, may not heal spontaneously and lead to nonunion prognosis due to the size of defects or unstable biomechanical properties, unfavourable wound environment, suboptimal surgical technique, metabolic factors, hormones, nutrition, and applied stress [2,3]. Bone grafts or substitute biomaterials are commonly used therapeutic strategies for clinical bone surgery to fill the bone defects for reconstructing large bone segments. Although autografts are the current gold standard treatment for bone defect regeneration [4,5], it still has disadvantages such as limitation in donor supply [6], donor site pain, or haemorrhage [7]. Other disadvantages of allograft are the risk of immune-mediated rejection, the transmission of infectious diseases and the negative effect on the mechanical and biological properties of graft [8—11]. In order to overcome the limitations associated with the current standard treatment of bone grafts, there has been an increasing interest in studying substitutes biomaterials, which are made of naturally derived and/or synthetic materials, during the past decades throughout the world [12—16]. The ideal bone graft substitutes should be biocompatible, bioresorbable, osteoconductive, osteoinductive, structurally similar to nature bone, and easy or ready to use.

Prior to testing in human beings, an ideal bone substitute should be tested both *in vivo* and *in vitro*, so as to make sure that it works effectively and safely. Therefore, to establish a suitable animal model is an indispensable step when evaluating the mechanical property and biocompatibility of bone substitute biomaterials. In this review, we discuss the speciality of different species for estimating bone defect substitute biomaterials in different bone defect sites, such as crania [17—19], femora [20—22], and ulna [23—25]. We evaluated the advantages and disadvantages of each species for estimating specific defects, analysed and compared the similarities between animal models and human clinical situations, and emphasised the factors we need to consider when choosing animals.

General selection criteria

A number of animal test models, such as rat/mouse [26—30], rabbit [31—34], dog [35—38], sheep [39—41], goat [42—44], and pig [45—48], have been developed to simulate human *in vivo* environment and physical conditions to test the availability and comparability of bone substitute biomaterials. In order to mimic various orthopaedic situations, many defect sites have been explored, such as calvaria [17—19], femora [20—22], and ulna [23—25]. A prerequisite for such a model is that no spontaneous complete osseous regeneration of the created defects occurs during the lifetime of the animals [49]. The critical size defect is defined as the smallest osseous wound that does not heal spontaneously over a long period of time. For practical purposes, if there is no mineralised area of $\geq$30% after 52 weeks, there would never be complete bony regeneration. Although the minimum size that renders a defect "critical" is not well understood, it has been defined as a segmental bone deficiency of a length exceeding 2—2.5 times the diameter of the affected bone [11,50].

Various factors have to be considered for selecting a specific animal species as a testing model. First and foremost, the chosen animal model should clearly demonstrate both significant physiological and pathophysiological analogies in comparison to humans. Second, it must be manageable to operate and observe a multiplicity of study objects postsurgery over a relatively short period of time [51]. Other selection criteria include costs for acquisition and care, animal availability, acceptability to society, tolerance to captivity, and ease of housing [52]. According to the international standard, we should also consider the size of the implant test specimens, number of implants per animal, intended duration of the test, and potential species' differences with regard to biological responses [53].

The following are the most frequently used animal models for creating bone defects to test conventional and innovative biological biomaterials to be used as bone substitutes.

Rabbits

Advantage and disadvantage of rabbit models

Rabbit is one of the most commonly used animal models, and it ranks first among all the animals used for musculoskeletal research [54]. However, regarding the assessment of multiple substitute biomaterials, the small size of rabbits is the major drawback for studying orthopaedic implants. However, it was reported that there were similarities in bone mineral density and the fracture toughness of mid-diaphyseal bone between rabbits and human [55]. Besides, in comparison with other species, such as primates or some rodents, rabbit has faster skeletal change and bone turnover [56]. Rabbits are easily available, and easy to house and handle. These characteristics make rabbits the first choice when researchers develop animal model for the *in vivo* test of a new bone substitute biomaterials.

Application of bone defect model for testing bone substitute biomaterials in rabbits

In recent years, several rabbit models have been used to test new bone substitute biomaterials. The most common implantation sites include bilateral tibiae and distal femur (Table 1). Walsh et al [57] investigated three commercially available and clinically used β-tricalcium phosphate (TCP) bone graft substitutes with the same chemistry (Vitoss, Osferion, Chronos), but with various macro- and microscopic characteristics, using a bilateral tibial metaphyseal defect model on New Zealand white rabbits. Bilateral defects (5 mm wide and 15 mm long) spanning the metaphyseal and

Table 1 Rabbit bone defect models for testing bone substitute biomaterials.

Defect site	Weight (kg)	Defect size	Substitute biomaterials
Tibiae	3—3.5	5 mm wide & 15 mm long [57], 6 mm in diameter [60,61]; 5 mm in length [77]	β-TCP bone graft substitutes [57]; hydroxyapatite 60%/B-tricalcium phosphate 40% [60]; porous titanium granules [61]; β-TCP, type I collagen, & rhFGF-2 [77]
Femur	3—5	7 × 10 mm^2 cylinder [59], 3 mm in diameter, 15 mm long [75]; 6 mm diameter × 5 mm cylinder [64,68]	Injectable calcium phosphate bone substitute [59]; PLGA/TCP/icaritin [75]; magnesium alloy AZ91D [63]; micro/ma-MCP [64]; CMMS/rhBMP-2 [65]; magnesium calcium phosphate biocement [66]; magnesium scaffolds [68]; magnesium silicate (m-MS) [67]; poly(epsilon-caprolactone)—poly(ethyleneglycol)—poly(epsilon-caprolactone) composite scaffolds [67]
Calvaria	2.0—3	10 mm diameter × 1.2 mm [78]; 9 mm diameter [79]	Apatite-coated zirconia [78]; low-molecular-weight silk fibroin [79]
Ulna	3.5—4	12 mm segment of midshaft ular [80]; 15 mm segment of midshaft ular [13]	PLGA/tricalcium phosphate/icaritin/BMP-2 scaffolds [80]; BMP-2/PLGA-coated gelatin sponge [13]

PLGA = poly(lactic-co-glycolic acid); TCP = tricalcium phosphate.

diaphyseal regions were created 3 mm below the joint line in the anteromedial cortex of the proximal tibia. It turns out that all three β-TCP bone graft substitutes performed well in this rabbit model. Young et al [58] developed an easily accessible and reproducible, nonhealing, alveolar, 10 mm "full-thickness" cylindrical defect removing both cortical plates and the intervening trabecular bone and tooth roots bone defect in the rabbit mandible. Gauthier et al [59] used a cylindrical, 7—10 mm critical-size bone defect rabbit model to investigate the efficiency of an injectable calcium phosphate bone substitute for bone regeneration. The critical-size bone defect rabbit model has been used successfully to carry out a histomorphometric analysis of a new, highly porous, biphasic calcium phosphate bone substitute by Calvo-Guirado et al [60]. Delgado-Ruiz et al [61] also used a critical-size tibiae defect rabbit model to test the behaviour of porous titanium granules, with and without membranes being covered. The test result showed that the porous titanium particles must be covered by a membrane, when grafting larger defects. Chen et al [62] established a critical-size bone defect model to test poly(lactic-co-glycolic acid) (PLGA)/TCP/icaritin 3D printing scaffold on the ulnar site.

Protocol for developing steroid-associated osteonecrosis rabbit model for testing bone substitute biomaterials

Distal femur defect rabbit model has commonly been used to test substitute biomaterials by a variety of researchers [59,63—68]. Although distal femur is not the commonly observed location of osteonecrosis in clinic, distal femur defects are frequently observed after the removal of malignant bone tumours [69] and total knee replacement [70—72]. Distal femur defects may lead to the failure of a total knee arthroplasty if left untreated [73]. Therefore, the use of a distal femur defect model is meaningful and constructive in testing substitute biomaterials prior to conducting human clinical trials. As the distal femoral defect rabbit model is one of the most commonly used animal models to test substitute biomaterials, here we review the surgical protocols for establishing steroid-associated osteonecrosis (SAON) rabbit models with distal femoral defect for testing bone substitute materials. SAON, which would subsequently lead to subchondral joint collapse, is caused by the frequent prescription of pulsed steroids as a life-saving agent in a situation such as severe acute respiratory syndromes. Core decompression is the major treatment method for SAON in the early stage. However, the nonunion bone defect that remained after the surgery may lead to insufficient mechanical support of the femur and result in joint collapse, seriously affecting the prognosis. Therefore, the use of suitable bone substitute materials to fill in the defect and provide a mechanical support to the femur is essential for the process of SAON bone defect repair. In order to test the substitute materials' biocompatibility, mechanical properties, and availability, a model of SAON is necessary.

The protocol for establishing a SAON rabbit model is based on our previously published work [74,75]. First of all, rabbits should be in healthy condition, weigh 3.0—3.5 kg, and reach an age of 28 weeks, which is similar to the adult age of human beings. Then, one injection of 10 mg/kg of lipopolysaccharide was given intravenously to the dopey rabbits. A day after that, three injections of 20 mg/kg of methylprednisolone were given intramuscularly at time intervals of 24 hours. Two weeks later, 93% of the rabbits developed osteonecrosis and none of them died during the procedure. This procedure is considered to be more effective or efficient, with a lower death rate compared with other published methods [76]. When the SAON rabbit model was established, we performed core decompression in rabbits by drilling a 3.0 mm tunnel transversely through the distal femora (Figure 1). Using this model, we have tested the *in vivo* bone defect repairing ability of a new bioactive PLGA/TCP composite scaffold incorporating the phytomolecule icaritin [75].

Assessment of bone regeneration is a key step to estimate the osteoconductive and osteoinductive abilities of bone substitute biomaterials. It is also an essential step for

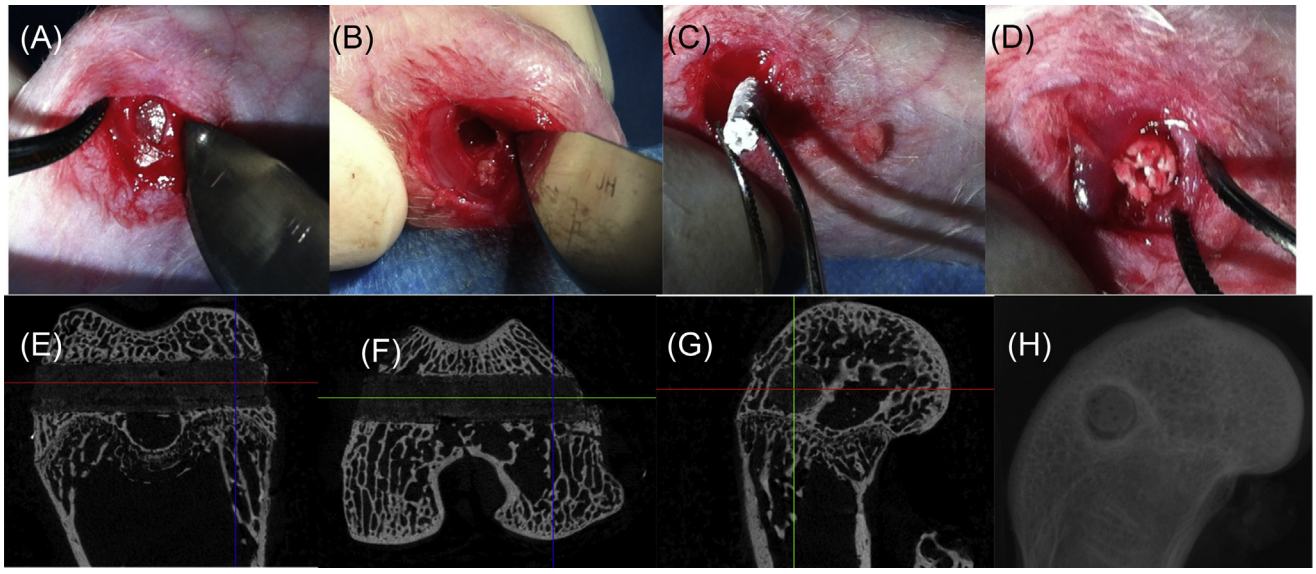

Figure 1 Surgical protocol for the establishment of core-decompression at the distal femur in a SAON rabbit model for implantation of the PLGA/TCP/icaritin substitute biomaterial. (A) The surgical site is exposed by an operating scalpel. (B) A 3.0 mm tunnel is drilled transversely through the distal femora by a trephine. (C and D) The biomaterial is implanted into the bone tunnel. (E—G) Micro-CT three-dimensional image of the bone defect site. (H) X-ray image of the bone defect site. CT = computed tomography; PLGA = poly(lactic-co-glycolic acid); SAON = steroid-associated osteonecrosis; TCP = tricalcium phosphate.

evaluating the function of a rabbit model. The most commonly used methodologies are histological analysis, microcomputed tomography (micro-CT) analysis, mechanical test analysis, radiograph analysis, sequential fluorescence labelling analysis, and dynamic contrast-enhanced magnetic resonance imaging. Using dynamic contrast-enhanced magnetic resonance imaging, we evaluated the effectiveness of the SAON rabbit model [74]. It has also been shown that using a SAON rabbit the *in vivo* osteogeneticability of PLGA/TCP/icaritin substitute biomaterial could be tested successfully [75].

Rodents

Anatomical advantages and disadvantages of rodents

As rodents are small in size and easy to handle, these are one of the most commonly used animal models, considered useful in preclinical studies for testing biomaterials as bone substitutes, and regarded as one of the first-choice models for *in vivo* test for regeneration of the bone tissue [81]. However, limitations of rodent models are also obvious. Compared with other larger animals such as rabbits, canine, and pigs, rodents have small-sized long bones and thin and fragile cortices [82]. Besides, rodent models do not show Haversian-type remodelling in the cortex, while lager animals do.

Application of rodent bone defect model for testing substitute biomaterials

Surgical implantation of substitute materials, such as β-TCP, calcium phosphate, and collagen, has been commonly conducted in rodents (Table 2). Kondo et al [83] investigated the biocompatibility of highly purified β-TCP bone graft substitutes using a rat femur defect model. Their study suggested that purified β-TCP was biocompatible and resorbable. In a study of 3D printing of composite calcium phosphate and collagen scaffolds for bone regeneration, a critical murine femur defect model was used to demonstrate the *in vivo* properties of substitute biomaterials [84]. Ye et al [85] established a 4-mm-diameter calvaria critical-size defect model in 6—8-week-old nu/nu mice. Based on this model, the efficacy of iPSCs/silk scaffold in increasing bone formation was revealed. Those rodent bone defect models have all been used successfully to test the *in vivo* osteoconductive and osteoinductive abilities of bone substitute materials.

Protocol for establishing calvaria critical-size defect nude mice model for testing bone substitute biomaterials

Critical-size defects are considered the smallest wounds established intraosseously, which cannot heal spontaneously during the lifetime of the animal [86]. In nude mice, a defect of 3 mm in size has been reported to be necessary to create a persisting femur bone defect [26]. Nude mice were anaesthetised according to the recommended routines for this species. A 4-mm-diameter calvaria critical-size defect was created on each side of the cranium using a dental bur attached to a slow-speed hand-piece with minimal invasion of the dura mater. Critical-size defects were created, which received implantation of substitute biomaterials later [85].

Assessment of bone regeneration was performed later by micro-CT scan and reconstruction, which showed that the

Table 2 Rodent bone defect models for testing new substitute biomaterials.

Defect site	Animal	Age/weight	Defect size	Substitute biomaterials
Distal femur	F344/Fisher [83]; male Wistar rat [87]	8 wk [83]; 12–14 wk [87]	2 mm diameter & depth [83]; 2 mm in diameter & 3 mm in length [87]	β-TCP bone graft substitutes [83]; polymer containing TGF-β1 [87]
Midfemur	Female BALB/cJ [84]; male Fisher [88]; nude rat [89]	13–15 wk [84]; 253 g [88]; 325–400 g [89]	2 mm in length [84]; 5 mm in length [88]; 5 mm in length [89]	Composite calcium phosphate & collagen [84]; marrow cells & porous ceramic [88]; BMP–silk composite matrices [89]
Calvaria	Nu/nu mice [85]; nude rat [90]	6–8 wk [85]; 12 wk [90]	4 mm diameter [85]; 8 mm diameter [90]	iPSCs/silk scaffold [85]; PLGA & adipose-derived stem cells [90]

BMP = bone morphogenetic protein; PLGA = poly(lactic-co-glycolic acid)

majority of the calvaria defects were filled with a substantial amount of newly formed bone tissue in the defect site treated with the SATB2-transduced iPSC implants 5 weeks postoperation. Histological analysis of bone regeneration further demonstrates radiographic results, indicating that the SATB2-transduced group showed nearly complete osseous closure of the defect [85].

Pigs

Anatomical advantage and disadvantage of pigs

Pigs are considered to be close representative models of human bone regeneration processes with regard to bone anatomy, morphology, healing capacity, remodelling, mineral density, and concentration [91,92]. Moreover, similarities have been found in the femur cross-sectional diameter and area between humans and pigs [93]. Besides, pigs also have a lamellar bone structure similar to that of humans [94]. However, pigs have a denser trabecular network, which considered intricate, difficult to handle, noisy, and aggressive; hence, pigs are often neglected in favour of more amenable species such as sheep and goats [95–97]. Furthermore, the length of the tibiae and femora in pigs is relatively small, which cannot meet the special implant needs of humans. Pig was the animal of choice for critical-size defect models to test bone substitute biomaterials because its bone regeneration rate (1.2–1.5 μm/d) is comparable to that of humans (1.0–1.5 μm/d) [49]. Commercial pigs are generally considered undesirable for orthopaedic research because of their large growth rates and very high body weight. However, the development of miniature pigs and micropigs has overcome this problem to some extent (Table 3).

Application of pig bone defect models for testing substitute biomaterials

Wehrhan et al [45] created a gene delivery method to increase bone formation in a porcine craniofacial bone defect model. The results showed that the gene delivery method formed more new bone in the defect site. Riegger et al [98] created circumscribed cylindrical bone defects of 11 mm diameter and 25 mm depth without penetration of the lateral cortex in the medial plateau of the tibia of 16 minipigs. The defect model was created to test the *in vivo* effect of the granular calcium phosphate composites and bone marrow aspiration concentrate. They found that there was a significant correlation between the two detective methods, showing that multidetector CT could be a promising tool for monitoring bone healing. A minipig infant model with craniofacial bone defect was created to test the *in vivo* effect of autologous bone grafts and bone morphogenetic protein-7 (rhBMP-7) by Springer et al [48]. Rohner et al [99] used a pig orbital defect model to show the *in vivo* efficacy of bone marrow-coated polycaprolactone scaffolds. Their studies showed that this bone marrow-coated 3D polycaprolactone scaffold is a

Table 3 Pig bone defect models used to test bone substitute biomaterials.

Animal	Defect site	Defect size	Substitute biomaterials
Porcine	Craniofacial	10 mm diameter & 10 mm depth	HA/TCP, PEG membrane, BMP-2 [45]
Göttinger minipigs	Tibial	11 mm diameter & 25 mm depth	Granular calcium phosphate, bone marrow aspiration concentrate; platelet-rich plasma [98]
Minipig	Parietal	2×4 cm^2	Particulate iliac bone graft, rhBMP-7 composite [48]
Pig	Orbital	2×2 cm^2	Bone-marrow-coated polycaprolactone scaffolds [99]

BMP = bone morphogenetic protein; PEG = poly(ethyleneglycol).

promising substitute biomaterial for enhancing bone regeneration.

Protocols for developing a porcine craniofacial bone defect model and testing substitute biomaterials

The porcine craniofacial bone defect model is used widely for testing bone substitute biomaterials. A commonly used protocol for developing a porcine craniofacial bone defect model and testing substitute materials was reported by Wehrhan et al [45]. Briefly, after anaesthetising domestic pigs and exposing the skull, nine defects of 10 mm diameter and 10 mm depth were created on it. Three testing groups, i.e., HA/TCP covered by poly(ethyleneglycol) (PEG) membrane, HA/TCP mixed with PEG matrix, and HA/TCP mixed with BMP-2 transfected hFOB cells and PEG matrix, were filled in three out of nine defects. The remaining six defects were filled with HA/TCP. After 2 weeks, 4 weeks, and 12 weeks, the animals were sacrificed and the os frontale was harvested for the following histological and immunohistochemical analyses.

Sheep/goats

Advantage and disadvantage of sheep or goats

It has been reported that adult sheep offer the advantage of possessing a body weight similar to adult humans, and having long bones of dimensions suitable for testing human implants and prostheses [96], which is not possible in small species such as rabbits and dogs. Sheep bones have similar macrostructure to human bones, but histologically, the bone structure of sheep is different from that of humans. In sheep, bone consists predominantly of the primary bone structure [100] in comparison with the largely secondary bone structure of humans [101]. Secondary bone remodelling in sheep does not take place until an average age of 7—9 years [96], while at 3—4 years of age they have a plexiform bone structure comprising a combination of woven and lamellar bones within which vascular plexuses are sandwiched [51]. Mature sheep have a significantly higher trabecular bone density and subsequently greater bone strength when compared to humans [51,102]. However, differences may change with location. Some researchers argue that sheep are still valuable models for human bone turnover and remodelling activity, although differences in bone structure were defined [103—105]. Sheep are shown to have a larger amount of bone ingrowth than humans; this is probably due to the greater amount of cancellous bone in the distal femur of sheep compared with humans [106].

Application of sheep bone defect models for testing substitute biomaterials

Maissen et al [107] used an ovine segmental defect model to investigate the influence of rhTGFβ-3 on mechanical and radiological parameters of a healing bone defect. The osteogenesis and remodelling effects of a biphasic synthetic bone graft material (Genex Paste; Biocomposites, Staffordshire, England), composed of calcium sulphate and β-TCP, on the healing of a sheep vertebral defect model was described in a canine model by Yang et al [39]. Zhu et al [40] developed a sheep vertebral bone defect model to evaluate the new bioactive materials and assessed the feasibility of the model *in vivo*. Reichert et al [41] developed a preclinical ovine model for tibial segmental bone defect repair by applying bone tissue engineering strategies. Lippens et al [42] used a 6-mm-size unicortical tibia defect goat model to evaluate the *in vivo* bone formation effect of an injectable polymerisable pluronic F127 hydrogel derivative combined with autologous mesenchymal stem cells. Kobayashi et al [108] used a 8-mm-diameter and 15-mm-deep sheep vertebral bone void model to investigate the histological properties of three formulations of calcium sodium phosphosilicate.

Protocols for developing a sheep tibia defect model for testing bone substitute biomaterials

The sheep tibia defect model has been used to test bone substitute by many researchers [41,42,107]. Here we review the protocol of an 18-mm-long mid-diaphysis tibia defect created in sheep for testing substitute materials and autologous bone graft. The defect was created in a 4—5-year-old sheep model and stabilized with a unilateral external fixator. The implant to the defects was divided into four groups. Assessment of *in vivo* stiffness was performed every week in a 4-week period by a custom-made device [107]. The radiology result revealed that only the bone graft group showed obvious recovery. Radiographic as well as computer tomographic evaluation was used to assess bone regeneration of the defect side.

Conclusion

Animal models play an indispensable role in testing bone substitute biomaterials for understanding their osteoconductivity, biocompatibility, mechanical properties, degradation, and interaction with host tissues. In this review, we summarised the most commonly and successfully used animal models, and the protocols that may be used as references to establish relevant preclinical experimental animal model(s) for testing both biosafety and treatment efficacy of bone substitutes (Table 4). After reviewing >100 publications about *in vivo* tests of biomaterials, we conclude that most authors fail to discuss the reason for choosing the animal model that they established and the clinical indication that they are stimulating. Although no animal model is perfect to simulate clinical conditions, we recommend that animal models should be established based on clinical indications. Finally, anaesthesia practice and specific surgical protocol should be included in the publications so as to make sure that animal welfare is well established.

Table 4 Summary of advantages and disadvantages of different bone defect animal models.

Animal species	Bone defect site	Advantages	Disadvantages
Pig	Craniofacial	Bone anatomy, morphology, healing capacity, & remodelling similar to humans; similar bone structure with respect to bone mineral density & concentration; a lamellar bone structure	Denser trabecular network, intricate & difficult to handle, noisy & aggressive, shorter tibiae & femur, large growth rates, & very high body weight
Sheep	Tibiae	Body weight similar to adult humans, easy to handle & house, relatively inexpensive, available in large numbers	Significantly higher trabecular bone density & subsequently greater bone strength, larger amount of bone ingrowth than humans
Rabbit	Tibiae femur	Easy to handle & small size, reaching skeletal maturity shortly after sexual maturity at ~6 mo of age	Small size; differences in bone anatomy, such as size & shape of the bones & also in loading; faster skeletal change & bone turnover
Rodent	Femur calvaria	Easy to handle & small size, life span suitable for postsurgery observation	Small-sized long bones & thin & fragile cortices, no showing of Haversian-type remodelling in the cortex

Conflicts and interest

The authors have no conflicts of interest to declare.

Acknowledgments

The authors are grateful for the financial support from NSFC-DG-RTD Joint Scheme (Project No. 51361130034), the European Union's 7th Framework Program under grant agreement No. NMP3-SL-2013-604517, NSFC grant (Project No. 51203178), and Shenzhen Fundamental Research Foundation (Project No. JCYJ20120617114912864).

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