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Standard Guide for Pre-clinical *in vivo* Evaluation in Critical Size Segmental Bone Defects[1]

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1. Scope

1.1 This guide covers general guidelines for the *in vivo* assessment of tissue-engineered medical products (TEMPs) intended to repair or regenerate bone. TEMPs included in this guide may be composed of natural or synthetic biomaterials (biocompatible and biodegradable) or composites thereof, and may contain cells or biologically active agents such as growth factors, synthetic peptides, plasmids, or cDNA. The models described in this guide are segmental critical size defects which, by definition, will not fill with viable tissue without treatment. Thus, these models represent a stringent test of a material's ability to induce or augment bone growth.

1.2 Guidelines include a description and rationale of various animal models including rat (murine), rabbit (leporine), dog (canine), goat (caprine), and sheep (ovine). Outcome measures based on radiographic, histologic, and mechanical analyses are described briefly and referenced. The user should refer to specific test methods for additional detail.

1.3 This guide is not intended to include the testing of raw materials, preparation of biomaterials, sterilization, or packaging of the product. ASTM standards for these steps are available in the Referenced Documents (Section 2).

1.4 The use of any of the methods included in this guide may not produce a result that is consistent with clinical performance in one or more specific applications.

1.5 Other pre-clinical methods may also be appropriate and this guide is not meant to exclude such methods. The material must be suitable for its intended purpose. Additional biological testing in this regard would be required.

1.6 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

1.7 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.*

2. Referenced Documents

2.1 *ASTM Standards:*[2]

F561 Practice for Retrieval and Analysis of Medical Devices, and Associated Tissues and Fluids

F565 Practice for Care and Handling of Orthopedic Implants and Instruments

F895 Test Method for Agar Diffusion Cell Culture Screening for Cytotoxicity

F981 Practice for Assessment of Compatibility of Biomaterials for Surgical Implants with Respect to Effect of Materials on Muscle and Bone

F1983 Practice for Assessment of Selected Tissue Effects of Absorbable Biomaterials for Implant Applications

F2150 Guide for Characterization and Testing of Biomaterial Scaffolds Used in Tissue-Engineered Medical Products

2.2 *Other Documents:*

21 CFR Part 58 Good Laboratory Practice for Nonclinical Laboratory Studies[3]

21 CFR 610.12 General Biological Products Standards—Sterility[3]

3. Terminology

3.1 *Definitions:*

3.1.1 *bone regeneration*—the formation of bone that has histologic, biochemical, and mechanical properties similar to that of native bone.

3.1.2 *bone repair*—the process of healing injured bone through cell proliferation and synthesis of new extracellular matrix.

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[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

[3] Available from U.S. Government Printing Office Superintendent of Documents, 732 N. Capitol St., NW, Mail Stop: SDE, Washington, DC 20401, http://www.access.gpo.gov.

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3.1.3 *compact bone*—classification of ossified bony connective tissue characterized by the presence of osteon-containing lamellar bone. Lamellar bone is highly organized in concentric sheets.

3.1.4 *cortical bone*—one of the two main types of osseous tissue. Cortical bone is dense and forms the surface of bones.

3.1.5 *critical size defect*—a bone defect, either naturally occurring or artificially created, which will not heal without intervention. In the clinical setting, this term applies to exceeding a healing period of approximately 6 months (in otherwise healthy adults).

3.1.6 *diaphyseal*—pertaining to the mid-section of long bones.

3.1.7 *endochondral ossification*—one of the two main types of bone formation, where a cartilaginous matrix forms first and is subsequently replaced by osseous tissue.

3.1.7.1 *Discussion*—Endochondral ossification is responsible for much of the bone growth in vertebrate skeletons, especially in long bones.

3.1.7.2 *Discussion*—The other main mechanism for bone formation is *intramembraneous ossification*, where osseous tissue is formed directly, without cartilaginous precursor; occurs mainly in the formation of flat bones (skull).

3.1.8 *growth plate*—the anatomic location within the epiphyseal region of long bones corresponding to the site of growth of bone through endochondral ossification.

3.1.8.1 *Discussion*—The growth plate in skeletally mature animals is fused.

3.1.9 *long bone*—bone that is longer than it is wide, and grows primarily by elongation of the diaphysis. The long bones include the femurs, tibias, and fibulas of the legs, the humeri, radii, and ulnas of the arms, the metacarpals and metatarsals of the hands and feet, and the phalanges of the fingers and toes.

3.1.10 *marrow*—soft, gelatinous tissue that fills the cavities of the bones. It is either red or yellow, depending upon the preponderance of hematopoietic (red) or fatty (yellow) tissue.

3.1.10.1 *Discussion*—Red marrow is also called myeloid tissue.

3.1.11 *matrix*—either the exogenous implanted scaffold or the endogenous extracelluar substance (otherwise known as extracellular matrix) derived from the host.

3.1.12 *metaphyseal*—pertaining to the dense end-section of long bones.

3.1.13 *remodeling*—a life long process where old bone is removed from the skeleton (bone resorption) and new bone is added (bone formation).

3.1.14 *residence time*—the time at which an implanted material (synthetic or natural) can no longer be detected in the host tissue.

3.1.15 *skeletal maturity*—the age at which the epiphyseal plates are fused.

3.1.15.1 *Discussion*—In rodents, skeletally mature animals are characterized by defined gonads.

3.1.16 *trabecular bone*—ossified bony connective tissue characterized by spicules surrounded by marrow space.

3.1.17 *weight-bearing versus non-weight bearing models*—weight bearing is the amount of weight a patient or experimental animal puts on the leg on which surgery has been performed, generally described as a percentage of the body weight.

3.1.17.1 *Discussion*—Non-weight bearing means the leg must not touch the floor (i.e., supports 0 % of the body weight).

3.1.17.2 *Discussion*—Full weight bearing means the leg can carry 100 % of the body weight on a step.

4. Significance and Use

4.1 This guide is aimed at providing a range of *in vivo* models to aid in preclinical research and development of tissue-engineered medical products (TEMPs) intended for the clinical repair or regeneration of bone.

4.2 This guide includes a description of the animal models, surgical considerations, and tissue processing as well as the qualitative and quantitative analysis of tissue specimens.

4.3 The user is encouraged to use appropriate ASTM and other guidelines to conduct cytotoxicity and biocompatibility tests on materials, TEMPs, or both, prior to assessment of the *in vivo* models described herein.

4.4 It is recommended that safety testing be in accordance with the provisions of the FDA Good Laboratory Practices Regulations 21 CFR 58.

4.5 Safety and effectiveness studies to support regulatory submissions (for example, Investigational Device Exemption (IDE)), Premarket Approval (PMA), 510K, Investigational New Drug (IND), or Biologics License Application (BLA) submissions in the U.S.) should conform to appropriate guidelines of the regulatory bodies for development of medical devices, biologics, or drugs, respectively.

4.6 Animal model outcomes are not necessarily predictive of human results and should, therefore, be interpreted cautiously with respect to potential applicability to human conditions.

5. Animal Models

NOTE 1—This section provides a description of the options to consider in determining the appropriate animal model and bone defect size and location.

NOTE 2—Research using these models needs to be conducted in accordance with governmental regulations and guidelines appropriate to the locale for the care and use of laboratory animals. Study protocols should be developed after consultation with the institutional attending veterinarian, and need appropriate review and approval by the institutional animal care and use committee prior to study initiation.

5.1 *Defect Size:*

5.1.1 A high proportion of fracture injuries in humans occur in long bones. Accordingly, defects created in long bones are commonly used for assessing bone repair/regeneration in animal models.

5.1.2 In principle, critical-size defects may be achieved in both metaphyseal and diaphyseal locations. For the purpose of this guide, only defects created in the diaphyseal section of long bones will be described.

5.1.3 Significant variability exists between animal species with respect to the size and weight of the animal, anatomy, and

gait thereby influencing kinetics, range of motion, and mechanical forces on defects. These factors influence bone architecture and structure. These factors play a significant role in the response to injury or disease of bone. The user should consider carefully the animal model that is appropriate for the stage of investigation of an implanted TEMP.

5.1.4 Mechanical load has been shown to affect bone repair. Amongst the mechanobiological factors, intermittent hydrostatic pressure and load-bearing stresses play an important role in modulating bone development and maintenance, as well as bone degeneration The impact of mechanical load extent or duration on the implanted TEMP, and surrounding native bone, varies depending on the anatomic site. The defect site chosen to evaluate implants should, therefore, factor the impact of mechanical load on the performance of the implant.

5.1.5 It is recommended that an appropriate species and anatomic site be chosen, that have dimensions sufficiently large to adequately investigate and optimize the formulation, design, dimensions, and associated instrumentation envisaged for human use, especially in late stages of development.

5.1.6 Larger animals may be more appropriate for studying repair in defects and locations that more closely approximate those in humans.

5.1.7 Larger defect dimensions generally require a method of fixation to secure the implant and thereby reduce implant dislocation. The method of implant immobilization can negatively impact both the surrounding host tissue and repair tissue. Accordingly, the difference in the design of the test TEMP in models which generally do not require fixation should be factored into the interpretation of results with respect to predictability of outcomes in larger animal models and humans requiring fixation.

5.1.8 For each species, a critical size defect is defined as the minimum defect dimension that the animal is incapable of repairing without intervention. The dimensions of critical defects generally differ for each species and should be considered carefully when designing the implant dimensions and method of fixation. As an empirical rule, the length of the defect (created by ostectomy) should at least be equal to 1.5 times the diameter of the selected bone (**1, 2**).[4] Some authors recommend at least 2 times the diameter of the selected bone (**3**).

5.1.9 Whether or not the periosteum from the resected segment of bone is still present can influence healing within the bone defect. The periosteum is typically removed in most studies of segmental critical-size defects. Whether or not the periosteum has been removed should be stated when reporting results.

5.1.10 Each study should include an empty-defect control group to confirm that the model is a critical-size defect. If/once the model is very well characterized, the use of historical data instead of actual control animals should be considered, in order to save on animal numbers, unless this would compromise the objectives of the study. For example, in pivotal preclinical proof-of-concept studies, concurrent controls are likely to be appropriate.

5.1.11 The use of unilateral defect models is generally recommended. This is especially true for weight-bearing locations in animals that use all four limbs for weight bearing (especially goats, sheep, and horses).

5.2 *Handling:*

5.2.1 Exposure of implants to extreme and highly variable mechanical forces as a result of jumping and running can lead to increased variability in outcome measures.

5.2.2 Potential differences in outcome when using weight-bearing versus non-weight bearing models should be carefully considered.

5.3 *Chromosomal Sex:*

5.3.1 Due to the impact of circulating steroids on cartilage and bone metabolism and regeneration, the choice of chromosomal sex should be considered. Animals in lactation should not be used. For some purposes, the use of aged or ovariectomized females (especially rats) may be indicated to simulate osteoporotic conditions.

5.3.2 It is recommended that the chromosomal sex be the same within the cohort, and that needs to be reported. The investigator should be aware that variances can occur between sexes and that appropriate statistical power needs to be instituted.

5.4 *Age:*

5.4.1 Bone undergoes dynamic changes in metabolism and remodeling during growth. Due to the impact of these physiologic processes on tissue repair, skeletally mature animals should be used. The cohorts should have fused epiphyseal growth plates. Skeletal maturity varies between species and can be determined radiographically if necessary.

5.4.2 Older animals have a greater propensity for osteopenia and have a decreased capacity to repair bone defects. If specific conditions are considered important for the intended TEMP assessment, then an appropriate model should be used.

5.4.3 The mesenchymal stem cell pool, growth factor responsiveness, and metabolic activity of cells generally decreases with age. Thus, reparative processes that are dependent on the number and activity of native cells may be partially compromised in older animals.

5.5 *Diet or Concurrent Pathology:*

5.5.1 In general, studies are performed with healthy animals under normal diet conditions. However, the addition of fluoride, as well as deprivation of vitamin D and/or calcium have been reported to mimic specific bone disease states. In situations where treatment of patients with systemic conditions that may affect bone repair is contemplated, non-clinical models that mimic the disease or conditions under consideration may be appropriate.

5.6 *Study Duration:*

5.6.1 The length of the study depends on the stage of TEMP development, the species used, the size of the defect, and the composition and design of the implant.

5.6.2 In rats and rabbits, small defects implanted for 8 to 12 weeks provide information regarding the residence time of the implant and fixation of the TEMP as well as the type of repair.

5.6.3 Using larger animals (dogs, sheep, goats), study periods of 8 to 12 weeks are limited to providing information

[4] The boldface numbers in parentheses refer to the list of references at the end of this standard.

regarding the biocompatibility, early cellular responsiveness, and the persistence and condition of the implant within the defect.

5.6.4 Periods of more than 3 months are generally necessary to gain confidence in the extent of success in the repair or regeneration of bone based on histologic outcome measures.

5.6.5 Depending on the study objective, it might be advisable to evaluate one or more cohorts in the study before full healing occurs. This may be of interest when comparing a new material with a standard material like autograft, where the difference between treatment groups may reach a transient maximum and then diminish over time. In general, it is necessary to match the claim and study end, taking into consideration the statistical power.

5.7 *Number of Animals:*

5.7.1 A statistically significant number of animals per group needs to be used. The required number depends on the intrinsic variability among the animals being used, the consistency of the surgical procedure which will be performed, the accuracy of the evaluation methods, the anticipated attrition rate of animals during the study, and the statistical techniques which will be used to analyze the data (**3**). Another important factor may be the objective of the study (for example, general feasibility/efficacy compared to an empty defect, or comparability of different constructs), and the variability of the treatment (for example, load of cells/factors, implant dimensions). The group size can be determined from existing data if the respective model is well established (literature or results from preliminary studies). For a pilot study, a group size of 6 to 8 is likely appropriate for histologic and mechanical testing as evaluation methods (**3**). For group sizes reported in the literature, see Appendix X1.

5.8 *Rat Model:*

5.8.1 Rats are among the most commonly used species for early-phase development, due to relatively low cost, housing space and ease of maintenance. The most commonly used model is a femoral defect (**3**).

5.8.2 Since the femur is a load-bearing location, the defect must be stabilized by internal or external fixation. Due to the small size of the animal, the fixation system may have to be custom-made. Plates (polymer, or metal) have been used with screws, K-wires/pins, and/or cerclage wire. Alternatively, external fixators have been described.

5.8.3 The typical defect size is 5 mm, created by a mid-diaphyseal ostectomy using a saw or dental burr. Care has to be taken to not injure the sciatic nerve during the procedure, as disuse of the operated leg can lead to delayed healing of the defect. For more details, see Appendix X1.

5.9 *Rabbit Model:*

5.9.1 The use of rabbits is generally more economical compared to larger species (dogs, sheep, or goats).

5.9.2 The thickness of cortical bone in rabbits is relatively less than in other species included in bone defect evaluations.

5.9.3 The rabbit radius is tubular, which may make it preferable for radiographic, histological, and mechanical evaluation.

5.9.4 A segmental critical size defect in either the ulna or the radius does not require a fixation, since the other bone will act as a stabilizer.

5.9.5 The typical defect size is 15 to 20 mm. Some studies caution that 15 mm is not large enough.

5.9.6 Adult rabbits with closed growth plates are preferred (more than approximately 20 weeks old). In younger animals, the intact bone of the operated leg can be overloaded, resulting in slipping of the growth plate and consequently exclusion from the study.

TABLE 1 Most Common Animal Model Parameters for the Assessment of Bone Repair in Critical Size Defects[A]

Species	Breed Commonly Used	Age of Adult Equivalency	Defect Sites Commonly Used	Typical Critical Size Defect Dimensions (mm)	Method of Fixation	Typical End Time-points	Evaluations
Rat[B] (Murine)	Sprague-Dawley, athymic nude, Fischer, Wistar, Lewis	6 months	F	5–10 mm	Polyethylene/ polyacetal plate with K-wires/ screws	8–24 weeks	Histology, radiographs/ Faxitron, biomechanics
Rabbit[B] (Lepus)	New Zealand White	9 months	R, U	20 mm	None (radius or ulna left intact)	8–12 weeks	Histology, radiographs, torsional strength
Dog[C] (Canine)	Beagle, Hound, Mongrel	>1–2 years	R, U, F	21–25 mm	Ex-fix, plate/ screws	12–24 weeks	Histology, radiographs, torsional strength
Goat[C] (Caprine)	Swiss Mountain	2–3 years	T	26–35 mm	Ex-fix	26 weeks	Histology, radiographs, compressive strength
Sheep[C] (Ovine)	Merino, Pre-Alpes, other	2–3 years	T, M	25–50 mm	Ex-fix, plate/ screws, intramedullary nail	16–24 weeks	Histology, radiographs, torsional or compressive strength

[A] For citations summarized in table, reference sections 5.7 – 5.11.
[B] Small animal.
[C] Large animal.

Legend: F, femur; T, tibia; R, radius; U, ulna; M, metatarsal

5.9.7 There is a certain risk in the rabbit ulna model that the radius can become attached to the defect area (fusion), which has to be considered if mechanical testing is one of the outcome measures. The axis of rotation for torsion testing becomes difficult to reproduce, and cross sectional area measurements are also difficult to make.

5.10 *Dog Model:*

5.10.1 Canines, such as medium-sized (for example, mean 10 to 15 kg) mongrels , beagles, and hounds have been used in critical-size defect models (**1**, **2**, **4-6**).

5.10.2 Long bones studied in canines have historically included the ulna, radius, and femur (**1**, **2**, **4-6**).

5.11 *Sheep Model:*

5.11.1 Sheep are commonly used for the study of bone healing in critical-size long-bone defects in large species animals.

5.11.2 The most common sites in the sheep are the mid-diaphyseal region of the metatarsal (**7-11**) and tibia (**12-18**).

5.11.3 Defects are typically 2 to 5 cm in the ovine tibia (**7-11**) and approximately 2.5 cm in the ovine metatarsal (**12-18**).

5.11.4 Defects in the sheep model are typically created unilaterally. Bilateral segmental diaphyseal defects in sheep are strongly discouraged.

5.12 *Goat Model:*

5.12.1 In comparison to sheep, goats are generally less averse to human interaction and are therefore easier to handle.

5.12.2 Goats should be screened by blood test for caprine encephalitis prior to inclusion in the cohort .

5.12.3 Critical-size defects in goats have been created unilaterally in the tibia (**19**). Bilateral segmental diaphyseal defects in goats are strongly discouraged.

6. Considerations for Defect Site

6.1 The focus of this guide is on mid-diaphyseal segmental defects in long bones.

6.2 Typical bones for the creation of mid-diaphyseal defects are the ulna, radius, tibia, fibula, and femur. Not all sites have been reported for all species.

6.3 Considerations should also include the level of difficulty of performing the surgical procedure and fixation.

6.4 Bilateral models of critical size segmental defects are generally considered contraindicated due to humane reasons and also possible effects on data integrity.

7. Considerations for Defect Type, Implant Fixation, and Joint Immobilization

7.1 *Joint Loading and Immobilization:*

7.1.1 The animal joint anatomy and joint size as well as gait should be taken into account to determine the appropriate immobilization modality.

7.1.2 Splints, external fixators, and casts can be used to reduce joint motion and loading for variable periods following surgery. There should be a point when the joint is restored to normal activity and exhibits unrestricted motion for an appropriate period of time.

7.1.3 The impact of disuse atrophy and potentially negative consequences to the bone should be considered when choosing the period of immobilization.

7.1.4 Continuous passive motion has been shown to provide some level of benefit to the regenerative process following bone injury in humans and animals. Implementation of similar therapeutic modalities in animal models is less feasible and has not been widely accepted.

7.1.5 The impact of limited access to surgical incision sites associated with the use of casts and splints should be factored into the postoperative care regime.

8. Test Procedures

8.1 *Implant Preparation:*

8.1.1 All materials to be implanted into animals should be verified to be non-cytotoxic and biocompatible. Implant components can be sterilized and prepared aseptically or end-point sterilized by methods known to be acceptable to the implant composition and function.

8.1.2 Bioburden or sterility testing, as appropriate, should be completed on representative test articles. Note that for TEMPs regulated as biologics in the United States, each lot must be tested for sterility in accordance with 21 CFR 610.12.

8.1.3 See Guide F2150, Practices F1983, F981, F565, and Test Method F895. Practice F1983 covers the assessment of compatibility of absorbable biomaterials for implant applications.

8.2 *Defect Generation:*

8.2.1 The defect should be created in a standard and reproducible manner.

8.2.2 Templates or other sizing tools should be considered, where feasible, for preparation of consistently sized defects.

8.2.3 Defects in all animals within a study should be created with the same type of tools and instruments.

8.3 *Test TEMP Implantation and Fixation:*

8.3.1 The test TEMP should be implanted in a standard and reproducible manner.

8.3.2 Care should be exercised to ensure that the surrounding bone is not excessively damaged and that the TEMP is in contact with the adjacent walls of the defect.

8.3.3 The defect should be fixed in a standard and reproducible manner.

8.4 *Recovery and Husbandry:*

8.4.1 Recovery conditions should be designed to reduce the potential for stress and excessive motion. For goats, sheep, and horses recovery pens that are sized to reduce excessive range of mobility for a period of two to three days are recommended.

8.4.2 All housing conditions should be approved by the United States Department of Agriculture (USDA), or the respective governmental agency of the country where the study is conducted.

8.4.3 Animals should be monitored frequently and observations recorded to ascertain appropriate health and physical condition.

8.4.4 A veterinarian should approve the health condition of animals prior to returning them to larger groups or herds.

8.5 *In-Life Period:*

8.5.1 The use of splints rather than standard dressings can reduce joint motion and loading; however, the impact of disuse atrophy and potentially negative consequence to the bone healing should be considered when choosing the length of treatment.

8.5.2 Radiographs should be used as appropriate for a given study to assess placement of the implants.

8.5.3 Following recovery, large animals should be contained within protected stalls for a minimum of nine days. After this period the animals can either remain in protected stalls or be allowed to roam freely in group herds.

8.5.4 A qualified veterinarian should examine animals routinely for any gross abnormalities or signs of discomfort.

8.5.5 Survival time should be designated based on the objective of the study. Typically, an early timepoint (for example, to examine the effect on early healing, including, for example, acceleration of healing), and one or two later timepoint(s) (for example, when full or nearly full healing is anticipated) are chosen. Historically used in-life periods are listed in the tables in Appendix X1.

8.6 *Necropsy:*

8.6.1 Animals should be euthanized in a humane manner according to accepted practices of the Animal Welfare Act (in the U.S.) or other applicable local statutes..

8.6.2 The implanted site should be removed along with the surrounding cartilage and bone.

8.6.3 Retrieved tissue should be placed in a solution consistent with intended outcome measures such as histology (decalcified paraffin versus nondecalcified plastic embedded), biochemistry, or mechanical testing.

9. Evaluation and Results

9.1 *Histology*—For histologic processing procedures, refer to Practice F561. Histological sections should be used to assess the amount and quality of tissue regeneration or repair within the defect. Histologic sections should be serially cut and stained in such a manner as to allow assessment of the quality of tissue and for detection of calcified tissue. Standard stains include: hematoxylin/eosin, Toluidine Blue, Modified Trichrome stain, and others (**3**). Consideration should be given to using decalcified versus undecalcified sections, which may require different staining methods.

9.1.1 *Microscopic Analysis and Scoring:*

9.1.1.1 Histologic sections should be analyzed for adverse tissue reactions using histopathologic indices.

9.1.1.2 For assessment of TEMP performance, a scoring system should be used to determine several aspects such as the following: callus formation, new bone formation in the defect (mineralized/non-mineralized), resorption of bone graft, cortex remodeling, marrow changes, union (distal, proximal) (for example, Ref **3**). In addition, fibrous connective tissue should be evaluated with regard to inflammation.

9.1.1.3 Histomorphometric analyses can be used to measure histologic parameters such as thickness, integration, cell number, and surface quality.

9.1.1.4 Time points of less than six months do not necessarily reflect the long-term outcome due to the potential for changes in the biochemical composition and organization of repair tissue over time.

9.1.1.5 Short-term histologic evaluation can be used for screening and optimization. Long-term assessment should be based on histologic and mechanical measures.

9.2 *Radiography:*

9.2.1 Radiographs are important to evaluate the amount and quality of the new bone forming during the in-life portion of the study, as well as at the endpoint.

9.2.2 Typically, radiographs should be taken in two orthogonal planes to allow assessment of proper alignment and a quasi-three-dimensional view.

9.2.3 Radiographic healing may be one of the decisive factors to terminate a study. It should be used in conjunction with other indicators, for example, clinical signs of full weight bearing.

9.2.4 Various radiographic scoring systems which take into consideration callus formation, bridging or union (proximal, distal), appearance of graft, and remodeling have been published. (**3**). The scoring system should be specified.

9.2.5 Inclusion of a metal wedge in the picture may help to normalize radiographs.

9.2.6 Radiopaque implants and fixation materials may have an impact on the ability to assess healing from radiographs.

9.3 *Computer Tomography:*

9.3.1 Computer Tomography (CT) has been evolving in recent years as a useful tool, for 3D imaging of bone regeneration in harvested bone, as well as for monitoring bone regeneration *in vivo* over time.

9.3.2 CT images to assess callus and bone (mineralized tissue) area are also useful for correct calculation and interpretation of mechanical test results.

9.3.3 The biggest challenge with CT analyses is to threshold appropriately to exclude the scaffold from newly forming bone within the defect.

9.4 *Mechanical Testing of Repair Tissue:*

9.4.1 Mechanical testing of the bone usually follows dissection. Care has to be taken when separating, for example, the ulna and radius if fusion is observed. Sample preparation may involve partial embedding into resin blocks to allow proper mounting in the fixtures.

9.4.2 Long bones are typically tested until failure.

9.4.3 Typical test setups are 3- or 4-point bending and torsional strength testing.

9.4.4 Consideration has to be given to the test speed, which should be reported.

9.4.5 From typical stress curves, the strength (maximum torque), stiffness, and total energy to failure can be calculated. It may also be helpful to report the angle at failure from torsional tests.

9.4.6 It is recommended to monitor and report where the fracture at failure occurs (in or through the newly formed bone tissue, or in the original bone outside the defect).

10. Analysis

10.1 *Statistical Analysis*—The mean and standard deviation should be calculated for the individual categories and the total

score for each of the graded specimens. Fisher exact test, chi-square test, or Kruskal-Wallis test (a one-way non-parametric analysis of variance) can be used for analyzing the differences between the scores of different groups.

11. Keywords

11.1 animal models; biomaterials; bone; bone regeneration; bone repair; defect generation; devices; implants; *in vivo*; mechanical testing; synthetic biomaterials; TEMPs (Tissue Engineered Medical Products)

APPENDIX

(Nonmandatory Information)

X1. PUBLISHED CSD MODEL EXAMPLES

TABLE X1.1 Published Examples for the Rat Femur Segmental CSD Model

Category	Yasko et al (20)	Chen et al (21)	Oakes et al (22)	Tsuchida et al (23)	Vogelin et al (24)	Jager et al (25)	Betz et al (26)
Citation	JBJS-A, 74(5), 659–670, 1992	J Orthop Res, 20(1):142–50, 2002	Clin Orthop Rel Res, 413: 281–290, 2003	J Orthop Res, 21(1):44–53, 2003	JSBS-A, 87(6):1323–31, 2005	Biomed Technik, 50(5):137–142, 2005	JSBS-A, 88A(2):355–365, 2006
Breed	Sprague-Dawley	Sprague-Dawley	Athymic or nude	Inbred Fischer 344; brown Norway	Lewis	Wistar; athymic rnu nude rats	Sprague-Dawley
Chromosomal sex	Male	male	Not specified	Male, female	Male	N/A	Male
Age	Adult	?	Not reported	Not reported	3 months	Adult	Adult
Weight	325–350 g	351–468 g	Not reported	90–240 g	345 ± 10.4 g	Not reported	400–425 g
Group size (n)	15	18 (3+3+12)	(90 total)	6–8	4 groups (10+10+15+15)	7(pilot); 12(pilot); 42	24+12+12
Size of defect	5 mm	6 mm	8 mm	6 mm	10 mm	4 mm	5 mm
Periosteum removed?	Not reported	Not reported	yes	N/A	Periosteal flap in 2 groups; otherwise, removed over 20 mm	yes	yes
Unilateral/bilateral	unilateral	unilateral	bilateral	unilateral	N/A	unilateral	unilateral
Fixation	Polyethylene plate, K-wires	Polyethylene plate; K-wires, cerclage wire	Plate/K-wire/ cerclage wire	Polyethylene plate; K-wires	Plate (6 hole, 1.5 mm)/screws	IM nailing (not recommended); external fixator	External fixator
Materials implanted	RhBMP-2 on inactive rat DBM	OP-1 + collagen (+ bacterial challenge?)	Human DBM putty in hyaluronic acid or glycerol carrier	Allogen. Mesench. SC	OPLA-HY	none	Adenovirus carrying rhBMP-2 gene
Implant volume	10 mg (reconstituted)	Not reported	Not reported	N/A	(10 mm × 3.7 mm diameter)	N/A	N/A
Duration of study	9 weeks	9 weeks	16 weeks	24 weeks	8 weeks	10 weeks	56 days (8 weeks)
Radiographs	1, 2, 3, 4.5, 6, 9 weeks	2, 4, 9 weeks	4, 8, 16 weeks	2, 4, 6, 8 weeks	4, 8 weeks	Every week	weekly
“Grading” of X-rays (quantification)	Scale 0–5 scale (5 = 100% healing)	(BioQuant workstation)	0–5 (5 = 100% healing)	5-point scale	No	No	3 categories (union, formation, no formation)
Histology	undecalcified, decalcified (multiple stains)	Formalin, dehydrated, embedded; 75-100 µm sections (hematoxylin/ eosin)	non decalcified (Toluidine blue)	(formaldehyde) demineralized, 5 µm sections (hematoxylin)	(Goldner-Masson trichrome)	No	(formaldehyde) demineralized (Hematoxylin, eosin; safranin O-fast green)
Biomechanical testing	yes	N/A	No (“reported elsewhere”)	N/A	N/A	N/A	5 rad/min
Empty control	Not reported	at 10 wk: 12/12 less than 9% new bone formation	36 limbs; 0 of 14 healed at 16 wks.	Not in this study		All empty defects	
Other assessments	Dynamic radioisotope bone-imaging	Not reported	Not reported	pQCT	histomorphometry	N/A	Histomorphometry, microCT, Dual x-ray absorptiometry

TABLE X1.2 Published Examples for Rabbit Forearm Segmental CSD Models

Ulna

Category	Cook et al (27)	Bostrom et al (28)	Smith et al (29)
Citation	JBJS – A, 76(6):827–38, 1994	Clin Orthop Rel Res. (327):272–82, 1996	J Controlled Release. 36(1-2), 183–195, 1995
Breed	New Zealand White	New Zealand white	New Zealand white
Chromosomal sex	Male	Male	Ulna: mixed; radius: male
Age	adult	6 months	Min. 6 months
Weight	4–5 kg	3.5–5 kg	Not reported
Group size (n)	30 total: n = 6 for control groups, 2-3 for dose-response groups	Total 50; n = 10	N = 10 per ulna group; N = 8 or n = 10 for radius groups;
Size of defect	1.5 cm	2.0 cm	20 mm ulna; 2.0 mm radius; 1.5 mm found to be not critical size.
Periosteum removed?	(yes)	Yes (also removed from radius)	Removed with bone segment (not reported)
Unilateral/bilateral	Bilateral (unilat. In 1 group)	bilateral	Ulna: bilateral
Fixation	No (not necessary, radius left intact)	No (radius left intact)	No
Materials implanted	Guanidine-extracted, insoluble rabbit DBM, with rhOP-1	RhBMP-2, with PLGA microparticles/ autologuous blood	RhBMP-2, with PLGA microparticles/ autologuous blood or CMC; ICBM
Implant volume	125 mg carrier	Approx. 550 µL = 479 ± 16 mg	
Duration of study	12 weeks	8 weeks	8 weeks ulna—8/12 weeks radius
Radiographs	Weekly	Biweekly-faxitron	Biweekly
"Grading" of X-rays (quantification)	no	5-pt. Scale: 0-4 (4 = 76-100%)	6-point scale (5 = 80-100%)
Histology	Non-decalcified (Basic fuchsin, toluidine blue; von Kossa, Goldner trichrome)	Non-decalcified (Hematoxylin, eosin; Goldner-Masson trichrome)	Decalcified (Hematoxylin, eosin.)
Biomechanical testing	(never frozen) Torsional to failure (50 mm/min)	(frozen) torsional to failure; defect analysis classification	Not reported
Empty control group	Not reported	No	Not used; but for carrier alone in 15 mm defect (radius): 5/8 healed ≥ 20 mm defect size needed
Other assessments	Not reported	Not reported	Not reported

Radius

Category	Wheeler et al (30)	Brekke et al (31)	Mackenzie et al (32)	Geiger et al (33)
Citation	J Biomed Mat Res. 43(4):365–373, 1998	J Biomed Mat Res. 43(4):380–98, 1998	Plastic & Reconstr Surg. 107(4):989–96, 2001	J Bone & Mineral Res 20(11):2028–35, 2005
Breed	New Zealand white	Not reported	New Zealand white	New Zealand white
Chromosomal sex		Not reported	Male and female	female
Age	(skeletally mature)	Not reported	(skeletally mature)	6-9 months
Weight		Not reported	Not reported	3.1–5.8 kg
Group size (n)	N = 8 (8 groups)	Not reported	N = 6 (total: 48); historical controls n = 8	N = 12 (total: 60)
Size of defect	20 mm	20 mm	20 mm	15 mm
Periosteum removed?	Not reported	no	Not reported	yes (5 mm from each side)
Unilateral/bilateral	unilateral	Not reported	unilateral	Unilateral
Fixation	No	No	No	no
Materials implanted	Segmental autograft; rhBMP-2/ PLA	OPLA+hyaluronic acid+morphogen (rhBMP-2)	Rabbit DBM; + human fibrin; + human fibrin/rhFGF-1; (controls)	"GAM" (gene-activated matrix; collagen sponge)+ VEGF-DNA
Implant volume	Cylindrical, (4.68 x 20 mm)	Cylindrical, (4.68 x 20 mm) = 344 mm^3	150 mg DBM; 20 mm long cylinder	15 x 5 x 5 mm
Duration of study	4, 8 weeks	8 weeks	4, 8 weeks	6 or 12 weeks
Radiographs	Biweekly	Yes	biweekly	3, 6, 12 weeks
"Grading" of X-rays (quantification)	standardized		Standardized; "% radioopacity"	Step wedge for semiquantitative evaluation
Histology	No	Yes (Von-Kossa)	non-decalcified (Goldner-Masson trichrome; von Kossa)	Decalcified (Alcian blue; anti CD31 (immuno- localization))
Biomechanical testing	(frozen) torsional; 180 deg/s	Not reported	No	No
Empty control group	Yes, n = 8; non-union (8/8)	Not reported	Not reported	Not reported
Other assessments	Not reported	Not reported	histomorphometry	Histomorphometry; microCT

TABLE X1.3 Published Examples for Canine Segmental CSD Models

Category	Bruder, et al (1)	Cook, et al (2)	Itoh, et al (4)	Sciadini, et al (5)	Arinzeh, et al (6)
Citation	JBJS-A, 80-A(7): 985–996, 1998	J Ortho Trauma, 12(6): 407–12, 1998	J Vet Med Sci, 60(4): 451-58, 1998	J Ortho Res, 18(2): 289–302, 2000	JBJS-A, 85A(10): 1927–35, 2003
Breed	Hounds	Mongrel	Beagles	Mongrel	Coonhound
Chromosomal sex	Female	Male	Male (3), female (6)	Male (18), female (9)	Not reported
Age	Adult	2–4 years	1.8-2.5 yrs (mean 2.7)		
Weight	20.3 ± 1.1 kg (mean)		9.2-14.8 kg (mean 12.9)	21-7–38.8 kg	22–25 kg
Group size (n)	15	16 dogs	8 dogs (4/group)	27 dogs (3 or 5/group)	12 dogs
Size of defect	2.1 cm	2.5 cm (≥ 1.5D)	2 cm	2.5 cm	2.1 cm
Periosteum removed?	Yes	Yes	Yes	Yes	Yes
Unilateral/bilateral	Unilateral femur	Bilateral ulna	Bilateral ulna	Bilateral radius	Unilateral (Not reported) femur
Fixation	Lengthening plate (Synthes)	None (radius intact)	Bone plate	External fixator	Lengthening plate (Synthes)
Materials implanted	MSCs on HA/TCP versus HA/TCP alone or no treatment	rhOP-1 (28), collagen only (2), no implant (2)	BMP2 + PLGA/ collagen gel (0,40,160,640 µg)	Collagen + BMP-2 (0, 150, 600, 2400 µg) versus canc autograft	MSCs on HA/TCP (versus historic controls)
Implant volume	Hollow tubes: 14 mmOD x 21 mmL	3.5 mL	1.6 cm^3	3 cm^3 (grp 1-4); 1.33 (grp 5), 6 cm^3 (grp 7)	Hollow tubes: 14 mmOD x 21 mmL
Duration of study	16 weeks	12 weeks	16 weeks	12, 24 wks; 48 wks (1)	16 weeks
Radiographs	4, 8, 16 wks	0, 2, 4, 6, 8, 12 wks		0, 2, 4, 8, 12, ...24 wks	4, 8, 16 wks
"Grading" of X-rays (quantification)	N/A	Scale 0-6	Xray absorptiometry (bone density), 0-5	Density (Microimages/ image analysis)	N/A
Histology	Non-decalcified tol blue or MacNeal light green, histomorph	Nondecal- 50 µm, 5 µm sections, basic fuschin & tol blue	Decalcified H&E (no histomorph)	Nondecalcified, sagittal and longitud, modified tol blue	Decalcified and nondecalc tol blue, histomorphometry
Biomechanical testing	N/A	Torsion to failure (w/in 8 hrs), 50 mm/ min, 6 cm lever arm	Not reported	Torsion to failure after fixation in formalin , 50 mm/min, 6 cm lever	N/A
Empty control?	Yes (3). No union; minimal bone, only at cut edges at 16 wks	Yes (2). Minimal bone-edges only; fibrous infill	No	No	No. Used historical empty control (Bruder, JBJS-A, 1998)
Comments		All returned to normal weight-bearing function			

TABLE X1.4 Published Examples for Goat Segmental CSD Models

Category	Buma, et al (34)	Dai, et al (19)
Citation	Biomaterials, 25(9): 1487–95, 2004	Calcif Tiss Int, 77: 55–61, 2005
Breed	Goats	Goats
Chromosomal sex	Not reported	
Age	Not reported	1 year
Weight	Not reported	18.6–31.5 kg
Group size (n)	Not reported	26 goats (9/6/6/3/2)
Size of defect	3.5 cm	2.6 cm
Periosteum removed?	No	Yes
Unilateral/bilateral	Not reported	Unilateral (R) tibia
Fixation	IM rod, screws	Circular external fixator
Materials implanted	Not reported	Biphasic calcined bone + MSCs ± transduced BMP-2
Implant volume	Not reported	16 mm OD x 25 mm L (7 mm ID)
Duration of study	26 weeks	8 weeks (n = 1/group), 26 weeks
Radiographs	Not reported	4, 8, 16, 26 weeks
"Grading" of X-rays (quantification)	Not reported	Compared to implants in mice
Histology	Not reported	Decalcified, H&E
Biomechanical testing	Torsion to failure	Compressive strength of explant cylinder
Empty control	Not reported	Yes (2). No union; minimal bone form'n
Other assessments	Not reported	Not reported

TABLE X1.5 Published Examples for Sheep Segmental CSD Models

Category	Gao, et al (12)	Gugala, et al (13)	Marcacci, et al (35)	Kon, et al (14)
Citation	Acta Orthop Trauma Surg, 116: 290–294, 1997	J Ortho Trauma, 13(3): 187–195, 1999	Calcif Tissue Int, 64: 83–90	J Biomed Matls Res, 49: 328–337, 2000
Breed	Sheep	Swiss sheep	Italian ‘massese’ sheep	Italian massese sheep
Chromosomal sex	Not reported	Female	Female	Female
Age	Adult	6–7 years	2 years	2 years
Weight	Mean 51 kg (41–66 kg)	50–60 kg		
Group size (n)	19 (11/8)	18 sheep (3/group)	5 sheep (1/group)	4 sheep (2/group)
Size of defect	2 cm	4 cm	3.5 cm	3.5 cm
Unilateral/bilateral	Unilat (left tibia only)	Bilateral(Not reported) tibia	Unilateral (left tibia only)	Unilat (left tibia only)
Periost removed?	not reported	Yes	Yes	(same as Marcacci)
Fixation	External rings + K-wires (11), autocompression plate (8)	AO external fix. + Steinmann pins	External (VM-Jet CiTiEffe, Italy)	External (VM-Jet CiTiEffe, Italy)
Materials implanted	TCP spacer (11), nothing (8)	PLGA membranes or CBM (auto/allo)	Porous, hollow HA cylinders	HA cylinders w/ & w/o MSCs
Implant volume	Not reported (cylinders 2 cm L)	Not reported	(35 mm x 20 mm D; 10 mm cenral bore)	
Duration of study	16 weeks	16 weeks	Up to 270 days	8 weeks
Radiographs	3, 6, 12, 16 wks post-op	0, every 2 wks	0, every 20 days	0, every 20 days; Post-op contact radiographs
“Grading” of X-rays (quantification)	Displ index (DI) versus max lateral	Not reported	Not reported	Not reported
Histology	Undemin (van Gieson) and demin (Alcian blue)	Decalcified and nondecalcified, Giemsa & eosin	Decalcified (H&E, Mallory, PAS, Alcian blue)	Nondecalcified sections; image analysis-% bone
Biomechanical testing	N/A	—	No	Indentation
Other assessments		CT (8, 10,…16 wk)	microrad	Gross exam, microrad, SEM
Empty control?	Yes. No union in any control defects (6) at 16 weeks	No. But no union in any defects without auto/ allograft	no	No. Control = HA cylinders w/o cells; some bone growth

Category	Blokhuis, et al (15)	Petite, et al (7)	Gugala and Gogolewski (16)	Viateau, et al (9)
Citation	J Biomed Matls Res, 51(3): 369–375, 2000	Nature Biotechnol, 18: 959–63, 2000	Injury, 33: SB71–SB76, 2002	Amer J Vet Res, 65: 1653–1657, 2004
Breed	Sheep	Sheep	Swiss mountain sheep	Pre-Alpes sheep
Chromosomal sex	Female	Not reported	Not reported	Female
Age	Not reported	Not reported	Not reported	2 years
Weight	50–75 kg	Not reported	Not reported	Mean 60 kg
Group size (n)	31 sheep (n = 8 or 7/group)	Not reported	3/group	18 sheep (3/3/5/7)
Size of defect	3 cm	6, 12, 15, 25 mm long	4 cm long	0.5, 1, 1.5, 2 x diaph diam (mean 6, 13, 25, mm)
Unilateral/bilateral	Unilateral tibia	metatarsal	Tibia (unilateral)	Left metatarsals III & IV
Periost removed?			Y (on bone segment)	
Fixation	AO unreamed tibial nail, locking bolts	Plate/ 6 screws	AO external fixator	Dynamic compression plate
Materials implanted	Calcium phosphate, CP/BMA, autograft, empty	Coral w/ and w/o cells	Membranes in (6) configurations; 2 groups included autograft	Nothing, autograft (group 4)
Implant volume	Not reported	Not reported	Not reported	Not reported
Duration of study	12 weeks	16 weeks	16 weeks	16 weeks
Radiographs	12 wks (contact)	4, 8, 12, 16 weeks	Weekly thru 16 weeks	0, 4, 8, 12, 16 weeks
“Grading” of X-rays (quantification)	0 (none) -4 (bridging all sides)			Qualitative; meas bone apposition in microrad
Histology	Undecalcified, tol blue & Goldner’s trichrome	Undecalcified; cortical and medullary bone area	Undecal; qualitative	Nondecalcified; Stevenal blue
Biomechanical testing	Torsion to failure	N	—	—
Other assessments	QCT, DEXA			
Empty control?	Yes. Greater torsional stiffness versus CP particles, CP + marrow	Y. No bone union. Minimal osteogenesis.	No	Yes (3 groups). No union in any control defects. Bone resorption and fibrous tissue seen in all

TABLE X1.5 Published Examples for Sheep Segmental CSD Models *(continued)*

Category	Zhangua, et al (8)	Regauer, et al (18)	Viateau, et al (10)	Pluhar, et al (17)	Viateau, et al (11)
Citation	J Huazhong Univ Sci Technol, 24(10): 62–67, 2004	Bone, 38(4): 564–570, 2006	Veterinary Surg, 35: 445–452, 2006	JBJS-Br, 88B: 960–966, 2006	JOR, 25: 741–749, 2007
Breed	Sheep	Merino sheep	Pre-Alpes sheep	Sheep	Pre-Alpes sheep
Chromosomal sex	Not reported	Not reported	Female	Female	Female
Age	2 ± 0.5 years	Adult	2 years	Skeletally mature	2 years
Weight	35 ± 10 kg		Mean 60 kg	Mean 70 kg	Mean 60 kg
Group size (n)	20 sheep (8/8/4)	19 sheep	11 sheep	26 sheep (10/10/6)	21 sheep
Size of defect	2.5 cm long	5 cm	2.5 cm	5 cm	2.5 cm
Unilateral/bilateral	Unilateral metatarsal	Unilateral tibia	Left metatarsal	Unilateral tibia	Unilateral metatarsal
Periost removed?	Not reported	Not reported	Yes not reported	Yes	Yes
Fixation	Not reported	IM rod	Compression plate, cast (first surgery)	Intramedullary nail	Compression plate
Materials implanted	Porous HA w/ and w/o MSCs, or empty	OP-1/deactivated DBM, w/ or w/o autograft, autograft alone	PMMA spacer for 6 wks, then autograft or empty	Coll/OP-1; Coll/OP-1/ CMC; empty	PMMA spacer for 6 wks; then autograft, granules w/ or w/o cells, or empty
Implant volume	Not reported		~4000 mm^3		
Duration of study	6, 12, 24 weeks	12 weeks	6 mos after 2nd surgery	4 months	6 mos after 2nd surgery
Radiographs	6, 12, 24 weeks	Faxitron, 12 wks	Not reported	Monthly (5/group)	Monthly
“Grading” of X-rays (quantification)	0-4 qualitative scale; meas. callus thickness	Not reported	Not reported	Not reported	Not reported
Histology	Undecalcified; histomorphometry	Undecalcified, SAM (scanning acoust microsc)	Decal-H&E, Masson’s, etc; immuno- Col I, CBFa (preOBs), CD14 (macs), RANK (preOCs)	Not reported	Undecalcified-Stevenolo blue, Van Gieson picro-fuschine
Biomechanical testing	Compressive strength (at 10 N/mm load)	Not reported	Not reported	Torsional strength, modulus, stiffness (100 Hz)	Not reported
Other assessments	Not reported	Not reported	CT	Not reported	CT
Empty control?	Yes (4). “Non-healing” at 24 wks	No	Yes. No bone union; bone deposition only close to transected cortices	Yes. No fusion; 60% fibrous infill. Mech strength signif lower than treated defects	Yes. Bone growth only at cut edes. Defect filled with fibrous tissue

REFERENCES

(1) Bruder, S. P., Kraus, K. H., Goldberg, V. M., and Kadiyala, S., “The Effect of Implants Loaded with Autologous Mesenchymal Stem Cells on the Healing of Canine Segmental Bone Defects,” *Journal of Bone and Joint Surgery–American* , Vol 80-A, No. 7, 1998, pp. 985–996.

(2) Cook, S. D., Salkeld, S. L., Brinker, M. R., Wolfe, M. W., and Rueger, D. C., “Use of an Osteoinductive Biomaterial (rhOP-1) in Healing Large Segmental Defects,” *Journal of Orthopaedic Trauma*, Vol 12, No. 6, 1998, pp. 407–412.

(3) An, Y. H., and Friedman, R. J., eds., *Animal Models in Orthopaedic Research*, CRC Press LLC, Boca Raton, FL, 1999.

(4) Itoh, T., Mochizuki, M., Nishimura, R., Matsunaa, S., Kadosawa, T., Kokubo, S., Yokota, S., and Sasaki, N., “Repair of Ulnar Segmental Defect by Recombinant Human Bone Morphogenetic Protein-2 in Dogs,” *J Vet Med Sci*, Vol 60, No. 4, 1998, pp. 451–458.

(5) Sciadini, M. F., and Johnson, K. D., “Evaluation of Recombinant Human Bone Morphogenetic Protein-2 as a Bone Raft Substitute in a Canine Segmental Defect Model,” *Journal of Orthopaedic Research*, Vol 18, 2000, pp. 289–302.

(6) Arinzeh, T. L., Peter, S. J., Archambault, M. P., Van Den Bos, C., Gordon, S., Kraus, K., Smith, A., and Kadiyala, S., “Allogenic Mesenchymal Stem Cells Regenerate Bone in a Critical-Sized Canine Segmental Defect,” *Journal of Bone and Joint Surgery–American*, Vol 85-A, No. 10, 2003, pp. 1927–1935.

(7) Petite, H., Viateau, V., Bensaid, W., Meunier, A., de Pollak, C., Bourguignon, M., Oudina, K., Sedel, L., and Guillemin, G., “Tissue-Engineered Bone Regeneration,” *Nature Biotechnology*, Vol 18, 2000, pp. 959–963.

(8) Zhanghua, L. I., Yan, Y., Wang, C., Xia, R., Zhang, Y., Zhao, Q., Liao, W., Wan, Y., and Lu, J., “Repair of Sheep Metatarsus Defects by Using Tissue-Engineering Technique,” *Journal of Huazhong University of Science and Technology*, Vol 24, No. 10, 2004, pp. 62–67.

(9) Viateau, V., Guillemin, G., Yang, Y. C., Bensaid, W., Reviron, T., Oudina, K., Meunier, A., Sedel, L., and Petite, H., “A Technique for Creating Critical-Size Defects in the Metatarsus of Sheep for Use in Investigation of Healing of Long-Bone Defects,” *American Journal of Veterinary Research*, Vol 65, No. 12, 2004, pp. 1653–1657.

(10) Viateau, V., Guillemin, G., Calando, Y., Logeart, D., Oudina, K., Sedel, L., Hannouche, D., Bousson, V., and Petite, H., “Induction of a Barrier Membrane to Facilitate Reconstruction of Massive Segmental Diaphyseal Bone Defects: An Ovine Model,” *Veterinary Surgery*, Vol 35, 2006, pp. 445–452.

(11) Viateau, V., Guillemin, G., Bousson, V., Oudina, K., Hannouche, D., Sedel, L., Logeart-Avramoglou, D., and Petite, H., “Long-Bone Critical-Size Defects Treated with Tissue-Engineered Grafts: A Study on Sheep,” *Journal of Orthopaedic Research* , Vol 25, 2007, pp. 741–749.

(12) Gao, T, J., Lindholm, T. S., Kommonen, B., Ragni, P., Paronzini, A., and Lindholm, T. C., “Stabilization of an Inserted Tricalcium Phosphate Spacer Enhances the Healing of a Segmental Defect in Sheep,” *Arch Orthop Trauma Surg*, Vol 116, 1997, pp. 290–294.

(13) Gugala, Z. and Gogolewski, S., “Regeneration of Segmental Diaphyseal Defects in Sheep Tibiae Using Resorbable Polymeric Membranes: A Preliminary Study,” *Journal of Orthopaedic Trauma*, Vol 13, No. 3, 1999, pp. 187–195.

(14) Kon, E., Muraglia, A., Corsi, A., Bianco, P., Marcacci, M., Martin, I., Boyde, A., Ruspantini, I., Chistolini, P., Rocca, M., Giardino, R., Cancedda, R., and Quarto, R., “Autologous Bone Marrow Stromal Cells Loaded onto Porous Hydroxyapatite Ceramic Accelerate Bone

Repair in Critical-Size Defects of Sheep Long Bones," *Journal of Biomedical Materials Research*, Vol 49, 2000, pp. 328–337.

(15) Blokhuis, T. J., Wipperman, B. W., den Boer, F. C., van Lingen, A., Patka, P., Bakker, F. C., and Haarman, H. J. T. M., "Resorbable Calcium Phosphate Particles as a Carrier Material for Bone Marrow in an Ovine Segmental Defect," *Journal of Biomedical Materials Research*, Vol 51, 2000, pp. 369–375.

(16) Gugala, Z., and Gogolewski, S., "Healing of Critical-Size Segmental Bone Defects in the Sheep Tibiae Using Resorbable Polylactide Membranes," *Injury*, Vol 33, No. (Suppl 2, 2002, pp. SB71–SB76.

(17) Pluhar, G. E., Turner, A. S., Pierce, A. R., Toth, C. A., Wheeler, D. L., "A Comparison of Two Biomaterial Carriers for Osteogenic Protein-1 (BMP-7) in an Ovine Critical Defect Model," *Journal of Bone and Joint Surgery–British* , Vol 88-B, 2006, pp. 960–966.

(18) Regauer, M., Jurgens, P., Budenhofer, U., Hartstock, M., Bocker, W., Burklein, D., Mutschler, W., Sader, R., and Schieker, M., "Quantitative Scanning Acoustic Microscopy Compared to Microradiography for Assessment of New Bone Formation," *Bone*, Vol 38, 2006, pp. 564–570.

(19) Dai, K. R., Xu, X. L., Tang, T. T., Zhu, Z. A., Yu, C. F., Lou, J. R., and Zhang, X. L., "Repairing of Goat Tibial Bone Defects with BMP-2 Gene-Modified Tissue Engineered Bone," *Calcified Tissue International*, Vol 77, 2005, pp. 55–61.

(20) Yasko, A. W., Lane, J. M., Fellinger, E. J., Rosen, V., Wozney, J. M., and Wang, E. A., "The Healing of Segmental Bone Defects, Induced by Recombinant Human Bone Morphogenetic Protein (rhBMP-2). A Radiographic, Histological, and Biomechanical Study in Rats," *Journal of Bone & Joint Surgery–American Volume*, Vol 74, No. 5, 1992, pp. 659–670.

(21) Chen, X., Kidder, L. S., Lew, W. D., "Osteogenic Protein-1 Induced Bone Formation in an Infected Segmental Defect in the Rat Femur," *Journal of Orthopaedic Research*, Vol 20, No. 1, 2002, pp. 142–150.

(22) Oakes, D. A., Lee, C. C., and Lieberman, J. R., "An Evaluation of Demineralized Bone Matrices in a Rat Femoral Defect Model," *Clin Orthop Rel Res*, Vol 413, 2003, pp. 281–290.

(23) Tsuchida, H., Hashimoto, J., Crawford, E., Manske, P., and Lou, J., "Engineered Allogeneic Mesenchymal Stem Cells Repair Femoral Segmental Defect in Rats," *Journal of Orthopaedic Research*, Vol 21, No. 1, 2003, pp. 44–53.

(24) Vogelin, E., Jones, N. F., Huang, J. I., Brekke, J. H., and Lieberman, J. R., "Healing of a Critical-Sized Defect in the Rat Femur with Use of a Vascularized Periosteal Flap, a Biodegradable Matrix, and Bone Morphogenetic Protein," *Journal of Bone & Joint Surgery–American Volume*, Vol 87, No. 6, 2005, pp. 1323–1331.

(25) Jager, M., Sager, M., Lensing-Hohn, S., and Krauspe, R., "The Critical Size Bony Defect in a Small Animal for Bone Healing Studies (II): Implant Evolution and Surgical Technique on a Rat's Femur," *Biomedizinische Technik.*, Vol 50, No. 5, May 2005, pp. 137–142.

(26) Betz, O. B., Betz, V. M., Nazarian, A., Pilapil, C. G., Vrahas, M. S., Bouxsein, M. L., Gerstenfeld, L. C., Einhorn, T. A., and Evans, C. H., "Direct Percutaneous Gene Delivery to Enhance Healing of Segmental Bone Defects," *Journal of Bone & Joint Surgery–American Volume*, Vol 88A, No. 2, 2006, pp. 355–365.

(27) Cook, S. D., Baffes, G. C., Wolfe, M. W., Sampath, T. K., Rueger, D. C., and Whitecloud, T. S. 3rd, "The Effect of Recombinant Human Osteogenic Protein-1 on Healing of Large Segmental Bone Defects," *Journal of Bone & Joint Surgery–American Volume*, Vol 76, No. 6, 1994, pp. 827–838.

(28) Bostrom, M., Lane, J. M., Tomin, E., Browne, M., Berberian, W., Turek, T., Smith, J., Wozney, J., and Schildhauer, T., "Use of Bone Morphogenetic Protein-2 in the Rabbit Ulnar Nonunion Model," *Clinical Orthopaedics & Related Research*, Vol 327, 1996, pp. 272–282.

(29) Smith, J. L., Jin, L., Parsons, T., Turek, T., Ron, E., Philbrook, C. M., Kenley, R. A., Marden, L., Hollinger, J., Bostrom, M. P. G., Tomin, E., and Lane, J. M., "Osseous Regeneration in Preclinical Models Using Bioabsorbable Delivery Technology for Recombinant Human Bone Morphogenetic Protein 2 (rhBMP-2)," *Journal of Controlled Release*, Vol 36, No. 1-2, 1995, pp. 183–195.

(30) Wheeler, D. I., Chamberland, D. I., Schmitt, J. M., Buck, D. C., Brekke, J. H., Hollinger, J. O., Joh, S. P., and Suh, K. W., "Radiomorphometry and Biomechanical Asessment of Recombinant Human Bone Morphogenetic Protein-2 and Polymer in Rabbit Radius Ostectomy Model," *Journal of Biomedical Materials Research* , Vol 43, No. 4, 1998, pp. 365–373.

(31) Brekke, J. H., Toth, J. M., "Principles of Tissue Engineering Applied to Programmable Osteogenesis," *Journal of Biomedical Materials Research*, Vol 43, No. 4, 1998, pp. 380–398.

(32) Mackenzie, D. J., Sipe, R., Buck, D., Burgess, W., and Hollinger, J., "Recombinant Human Acidic Fibroblast Growth Factor and Fibrin Carrier Regenerates Bone," *Plastic & Reconstructive Surgery*, Vol 107, No. 4, 2001, pp. 989–996.

(33) Geiger, F., Bertram, H., Berger, I., Lorenz, H., Wall, O., Eckhardt, C., Simank, H. G., and Richter, W., "Vascular Endothelial Growth Factor Gene-Activated Matrix (VEGF165-GAM) Enhances Osteogenesis and Angiogenesis in Large Segmental Bone Defects," *Journal of Bone & Mineral Research*, Vol 20, No. 11, Nov 2005, pp. 2028–2035.

(34) Buma P., Schreurs W., Verdonschrot N., "Skeletal tissue engineering – from in vitro studies to large animal models," *Biomaterials*, Vol 25, No. 9, 2004, pp. 1487–1495.

(35) Marcacci, M., Kon, E., Zaffagnini, S., Muraglia, A., Giardino, R., Rocca, M., Corsi, A., Benvenuti, A., Bianco, P., Quarto, R., Martin, I., Cancedda, R., "Reconstruction of Extensive Long Bone Defects in Sheep Using Porous Hydroxyapatite Sponges," *Calcified Tissue International*, Vol 64, 1999, pp. 83–90.

Histology

(36) Carson, F., *Histotechnology, A Self Instructional Text*, ASCP Press, Chicago, IL, 1997.

(37) Sheehan, D., and Hrapchak, B., "Bone," *Theory and Practice of Histotechnology* , McCrawHill Book Company, NY, 1960, p. 787.

(38) Callis, G. and Sterchi, D., "Decalcification of Bone: Literature Review and Practical Study of Various Decalcifying Agents, Methods, and Their Effects on Bone Histology," *J. Histotechnol.*, Vol 21, 1998, pp. 49–58.

(39) Luna, L., *Manual of Histologic Staining Methods of Pathology*, Armed Forces Institute, 1960.

(40) Sterchi, D., and Keefer, L., "Modified Mallory Aniline Blue Stain for Bone, Cartilage, and Other Connective Tissues," *Journal of Histotechnology*, 21, 1998, pp. 129–133.

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