Published in final edited form as:

Methods Mol Biol. 2010 ; 666: 285–307. doi:10.1007/978-1-60761-820-1_18.

Preclinical Methods for the Evaluation of Periodontal Regeneration In Vivo

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Abstract

For the determination of key factors or devices that promote periodontal regeneration, preclinical investigations using in vivo animal models are critical for evaluating the biological responses before human clinical trial testing. In this chapter, we provide an overview on the commonly used preclinical animals for the study of reconstructive procedures to promote bone and soft tissue repair of tooth-supporting periodontal defects. Steps are provided on the animal management for evaluation of outcome measures using descriptive histology, histomorphometry, three-dimensional imaging, and safety assessments. The use of these key measures of periodontal regeneration should aid investigators in the selection of appropriate surrogate endpoints to be utilized in the clinical arena, which are not practical or ethical in humans. These methods will prepare investigators and assist them in identifying endpoints that can then be adapted to human clinical trial planning.

Keywords

Periodontal regeneration; tissue engineering; bioactive molecules; animal surgery; rat; canine

1. Introduction

There are many critical steps involved in the appropriate design and implementation of investigations for determination of the safety and efficacy of regenerative devices and biologics for periodontal repair. The first phase is molecule discovery, performed via in vitro experiments based on biological plausibility. A variety of bioactive molecules and osteoconductive scaffolding matrices have been demonstrated to be effective to enhance cell activity such as proliferation, differentiation, and matrix biosynthesis (1–5). After confirming safety and efficacy within animals, the planning and design for human clinical trials can begin. Thus, animal experimentation is a necessary bridge from the laboratory to the clinic. In the preparation of any preclinical animal investigation, regulatory agencies (FDA, EMEA, etc.) or local animal welfare agencies must be consulted to determine the welfare and planning of studies prior to initiation. Most countries have their own animal experimental guidelines for animal welfare. Animals must be handled and managed in the most humane environment possible with minimal suffering or loss of life as the goal.

1.1. Common Animal Models Used in Periodontal Research

A variety of different animal models such as rats, dogs, and non-human primates have been used in periodontal regenerative studies (6). For periodontal regeneration studies, the rat

periodontal model has been frequently used (7–9). The rat model is quite valuable as a screening tool for regenerative molecule assessment due to cost effectiveness, ease of handling, etc.; however, the typical defect size is relatively small making visualization challenging, thus requiring the use of surgical microscopes for defect creation. Large animal models, such as the canine or the non-human primate, make a logical next step. The canine wound healing kinetics and tooth anatomy have many similarities to the human situation. Non-human primates are highly desirable to evaluate the safety and efficacy of new molecules because their anatomic and biologic features are very close to humans. However, their high cost and the strict regulations needed prevent them from being more highly utilized. One should choose the preferred animal model according to study requirements. Here we review current periodontal regenerative animal models with a focus on rats and canines with specific details on the methods required to perform surgery and evaluate outcome measures.

2. Materials

2.1. Animal Welfare Guidelines

Animal-based research has led to significant improvements in the quality of life for every human being; however, these advances must be the result of humane use and care of animals used for research and instruction. Every investigator should adhere to the Public Health Service Policy on Humane Care and Use of Laboratory Animals, incorporating principles from the Guide for the Care and Use of Laboratory Animals while executing any work with vertebrate animals. Individual institutions have specific guidelines utilizing policies put in place by the federal and state governments, and it is necessary to review these guidelines before initiating any effort in animal research. Proper maintenance of documentation and medical and surgical records is also an essential component of compliance with federal and institutional guidelines (*see Note 1*).

2.2. Surgical Model

Before performing any in vivo regenerative experiment, the specific animal model should be selected based on outcome. Some animal models may provide critical-sized defects, while others spontaneously heal and are considered as kinetic defects. Thus, it is very important to choose the appropriate model to effectively analyze the effects of bioactive molecules in a specific study. Rat, canine, and non-human primate models are most often used for these experiments. This chapter provides specific details pertaining to the most common periodontal regeneration models (rats and dogs) (*see Notes 2 and 3*).

2.2.1. Rat Periodontal Regeneration Model

1. Figure 18.1 illustrates a periodontal fenestration defect model in the rat.
2. For the rat periodontal fenestration defect (7–9), an extraoral buccal approach should be used. This model is widely employed and is accepted as an appropriate one to test periodontal regeneration in small animals. This model provides isolation from the oral environment, due to the extraoral approach, and thus can

prevent negative effects such as contamination and infection by saliva and other microorganisms.

2.2.2. Canine Periodontal Regeneration Model

1. Figure 18.2 illustrates a periodontal surgical model in the canine.
2. The dog is very commonly used as a large animal for periodontal research because it provides many similarities to humans, such as microbiological and periodontal pathogenesis characterization. Premolar teeth have two roots, allowing for creation of critical size, supra-alveolar furcation defects. Furthermore, dogs are generally friendly and cooperative, making care and management easy (10).

2.3. Delivering Devices/Biomaterials

1. Table 18.1 shows popular delivery vehicles including collagen and hydroxyapatite (*see Note 4*).
2. Bioactive molecules can also be delivered as polypeptides (11, 12), proteins (7, 13), in engineered cells (14), and/or gene vehicles (8).

2.3.1. Viral Vectors as a Delivery Vehicle of Biologic Factors—Gene therapy

generally refers to the process of transferring specific genetic sequences to host cells using viral or non-viral vectors in order to produce proteins, with the preferred strategy dependent upon the characteristics of the target site. Viral vectors are more commonly used in current preclinical studies based on their transduction efficiency (15).

1. Adenovirus, adeno-associated virus (AAV), and retrovirus are widely used due to their high infection efficiencies.
2. Adenovirus delivers the genetic material in the form of double-stranded DNA and exhibits transiently high transduction efficiency on both dividing and non-dividing cells. The delivered genetic material neither incorporates into the host genome nor induces apparent phenotype change of the infected cells (16).
3. AAV delivers the genetic material in the form of singlestranded DNA and is capable of infecting both dividing and non-dividing cells at a sustained, therapeutic-relevant concentration. Wild-type AAV can insert the genetic material at a specific site; however, recombinant AAV will not incorporate genetic material into the host genome (17). AAV causes lower immunogenicity than does adenovirus (18).
4. Retrovirus reverse transcribes the RNA genome into the viral DNA and stably integrates into the host genome, resulting in long-term and high-level expression of a transgene. However, retrovirus application may have limited clinical effects due to random incorporation into the host genome as well as infecting only actively dividing cells, making certain types of cells refractory to viral infection (19).

2.4. Biologic Factors

1. Among several growth factors, platelet-derived growth factor (PDGF) (20, 21), fibroblast growth factor-2 (FGF-2) (22, 23), amelogenin (24, 25), and bone morphogenetic proteins (BMPs) (7, 13, 26) are well known to have peri-odontal regeneration potential (*see* Table 18.1).
2. Combined use of growth factors and scaffolding material may maximize delivery efficiency (27).
3. Combinatory therapy using two (or more) growth factors can also be studied (20, 28).

2.5. Instrumentation

1. Initial incision: Surgical blade (#11, 15), periosteal elevator (Pritchard) (*see* **Note 5**).
2. Defect creation: Bur, low-speed, and high-speed handpiece with engine and chisel.
3. Small, sharp, hand instrument such as Gracey curette.
4. Wound closure: Needle holder (Crile-Wood), suture material (vicryl resorbable), scissors (LaGrange double curved), and metal staples.
5. Anesthesia; will vary with animal model.
6. Sterile, sanitizable, surgical area with proper ventilation.
7. External heat source(s).
8. Hot-bead instrument sterilizer.
9. Sterile, clean cages for post-surgery animal recovery.
10. Staple remover.
11. Tissue harvesting: Scissors, round disc, and low-speed engine for tissue harvesting.
12. Visualizing microscope. We use a SMZ 1000 (Nikon, Melville, NY).

2.6. Tissue Processing and Embedding

1. Fixation solution:
 - a. 10% Neutral buffered formalin.
 - b. 50–70% Ethanol.
2. Tissue processor (Autotechnicon; International Medical Equipment, Inc., San Marcos, CA).
3. Embedding material for decalcified sample preparation: paraffin and paraffin oven.
4. Embedding material for undecalcified sample preparation:

- a. Epoxy resin: EMbed 812 (Electronic Microscopic Sciences, Hatfield, PA) or another substitute of EPON812 (29).
 - b. PMMA (polymethylmethacrylate): Technovit 9100 NEW® (Electronic Microscopic Sciences, Hatfield, PA) or equivalent product.
 - c. Propylene oxide (Fisher Scientific Co., Pittsburgh, PA) and acetone (Fisher) are also required for epoxy resin and PMMA embedding process.
5. Cutting device for decalcified sample preparation: Microtome.
6. Cutting and grinding device for undecalcified sample preparation:
 - a. ISOMETRIC diamond saw blade.
 - b. EXAKT cutting and grinding system.
7. Plastic or glass slides.
8. Ethanol series (70, 80, 90, 95, and 100%) and xylene.
9. Mounting solution – Permount (Fisher).

2.7. Tissue Staining

2.7.1. Hematoxylin and Eosin Stain

1. Gill's hematoxylin 1 (Fisher).
2. Eosin–phloxine B (Fisher).

2.7.2. Methylene Blue Staining

1. Toluidine blue solution: 1 g Toluidine blue and 1 g sodium borate added to 100 mL distilled water and filtered.
2. Toluidine blue equivalent solution: Sanderson's™ Rapid Bone Stain (Surgipath) solution.

2.7.3. Modified Goldner's Masson Staining

1. Weigert's iron hematoxylin.
2. Ponceau–fuchsin solution: 0.75 g Ponceau de xyloidine, 0.25 g acid fuchsin, and 1 mL acetic acid added to distilled water to achieve the final volume of 100 mL.
3. Azophloxine solution: 0.5 g azophloxine and 0.6 mL acetic acid added to distilled water to achieve the final volume of 100 mL.
4. Light green solution: 1 g Light green and 1 mL acetic acid added to distilled water to achieve the final volume of 100 mL.
5. Phosphomolybdic acid–orange G solution: 3 g Phospho-molybdic acid and 2 g orange G dissolved in 500 mL distilled water and then added a crystal of thymol.
6. 1% Acetic acid.

2.7.4. von Kossa Stain

1. 1% Silver nitrate (preserve in dark).
2. 2.5% Sodium thiosulfate.
3. 1% Safranin O.

2.7.5. Immunohistochemical Stains

1. 10 mM Sodium citrate (pH~6).
2. 50 mM Tris-HCl buffer.
3. 3% H₂O₂ (in 50 mM Tris-HCl).
4. 1% Bovine serum.
5. Primary and secondary antibody.
6. 3,3'-Diaminobenzidine (DAB) staining solution (DAKO Corp.).
7. Gill's hematoxylin 1.

2.8. Analysis

1. Optical microscope with imaging analysis apparatus. A Nikon Eclipse E800 microscope fitted with a SPOT-2 camera (Diagnostic Instruments, Inc., Sterling Heights, MI) and Image Pro Plus software (Media Cybernetics, Silver Spring, MD) can be used.
2. Micro-CT for additional three-dimensional analysis. eXplore Locus SP and MicroView v.2.0 (Analysis Plus, GE Healthcare) can be used.

3. Methods

3.1. Rat Model

3.1.1. Pre-operative Surgical Preparations—Animals require an acclimation period of approximately 3 days to 1 week after arrival in a new housing facility. The surgical area should be conducive to an aseptic surgery and must not be used for any other purpose during the time of the surgery. Ideally, the surgical area can be located within the housing facilities, therefore limiting stress and potential health hazards to the animals. Disinfectants such as Sporidicin[®], Clorox[®], Clidox[®], Cide-Swipes[®], Virex[®], and Cidex[®] can be used to clean and disinfect the surgery area, although some may not be as effective at eliminating all contaminants.

Animals and instruments must also be prepared in a way to prevent contamination and ensure success of the survival surgery (*see* **Notes 6** and **7**). Surgeons should also undergo appropriate preparation including, but not limited to, hand washing, wearing sterile gloves, gowns, and masks for each animal's surgical procedures.

3.1.2. Anesthesia—Rats can be anesthetized under general anesthesia by ketamine and xylazine combination via intraperitoneal (IP) injection, lasting for 45–90 min (*see* **Note 8**).

To prolong anesthesia, supplement with one-third dose of ketamine only (Horton, Hugunin, Cotroneo). All animals should be provided an external heat source (e.g., recirculating water blanket, microwaveable heating packs, or self-regulating heating pad) in indirect contact with the animal to prevent hypothermia during the entire anesthesia and recovery period.

3.1.3. Surgery—After preparing the animal for surgery, a scalpel incision can be made from skin to the masseter muscle to expose the rat mandibular bone (buccal plate) (*see Note 9*). The target area is the distal root of the mandibular first molar. Buccal roots of the first and second molars can be included in the surgical defect (*see Fig. 18.1*). Using a round bur with high-speed instrumentation, one can create a bony defect of 3 mm × 2 mm × 1 mm size (*see Note 10*). The periodontal ligament, cementum, and superficial dentin can be removed by hand instrumentation. After applying test agent(s) into the created defect area, the muscle and skin are repositioned by sutures and/or surgical clips.

3.1.4. Post-surgical Management—Following surgery, rats may be administered analgesia for pain, and antibiotics for infection control within the surgical site. Analgesics such as buprenorphine (0.01–0.05 mg/kg subcutaneous or intraperitoneal) should be administered for at least 24 h after the periodontal defect surgery. Antibiotics can be dispensed via the water supply (*see Note 11*), although normal chow should be readily available (*see Note 12*). Animals should be treated and monitored according to the animal surgery guidelines given by their institution in accordance with regulations and in compliance with animal housing authorities. For example, if biohazardous materials, such as viral vectors, are applied, the animal must be kept in biohazard facility until viral shedding has completed (*see Note 13*).

3.1.5. Sacrifice and Harvesting—At study endpoints, animals can be sacrificed and tissues harvested for analysis. Experimental endpoints are determined by previous studies, published literature, and/or may be based on the biological material properties themselves. If the molecules of interest act early in the healing process, the endpoints should be selected accordingly in order to capture important healing events. Euthanasia methods, such as CO₂ asphyxiation, should be selected according to institutional guidelines. A secondary method should be employed in order to confirm animal death prior to resuming tissue collection. Harvested samples should be fixed to prevent degradation without damaging the tissues.

3.2. Canine Model

3.2.1. Pre-operative Surgical Preparations

1. Animals require an acclimation period of approximately 3 days to 1 week after arrival in a new housing facility. The surgical area should be easily sanitizable and undergo careful preparation to ensure that aseptic surgical technique is carried out according to government and institutional guidelines. Ideally, the surgical area can be located within the housing facilities but should be separated from human occupancy areas, therefore limiting stress and potential health hazards to the animals. Disinfectants such as Sporocidin[®], Clorox[®], Clidox[®], Cide-Swipes[®], Virex[®], and Cidex[®] can be used to clean and disinfect the surgery area, although some may not be effective at eliminating all contaminants.

2. Animals and instruments must also be prepared in a way to limit or prevent contamination and ensure success of the survival surgery (*see Notes 7 and 14*). Surgeons should undergo appropriate preparation in a room separate from the operating areas, including, but not limited to surgical staff, wearing sterile gloves, gowns, caps, shoe covers, and masks for each animal's surgical procedures.
3. The pre-surgical oral hygiene phase is especially necessary for canine and non-human primates to obtain a healing response following surgery by ensuring biofilm removal and to begin with healthy periodontal tissues. Scaling and root planing procedures should be performed in an area separate from the surgical operating room to prevent contamination and can be carried out approximately 10 days prior to surgery.

3.2.2. Anesthesia—Prior to anesthesia, canines greater than 10 weeks of age should be fasted for at least 6 h, with the exception of water. Canines should be sedated using a combination of buprenorphine, acepromazine, and glycopyrrolate as a pre-sedative approximately 30–60 min prior to propofol administration for induction (*see Note 15*). The canine will then be intubated and maintained under general anesthesia with isoflurane delivered through a volume-regulated aspirator (13). Intravenous fluids, such as Ringer's solution (10 mL/kg/h), should be administered during surgery (*see Note 16*). Local infiltration anesthesia, for example, lidocaine HCl with epinephrine is helpful to limit bleeding at and near to the surgical site. All animals should be provided an external heat source, preferably a recirculating water blanket, to prevent hypothermia during the entire anesthesia and recovery period.

3.2.3. Surgery

1. *Defect creation*: Periodontal defects can either be created surgically (13, 30, 31), involve ligatures around teeth (32, 33), or develop naturally (34, 35). For surgical creation, defects can be made to the same size or shape to compare test groups and controls. The defects are typically created around the second, third, and fourth mandibular premolar teeth. After sulcular incisions and mucoperiosteal flap elevations, alveolar bone is removed from around the teeth to make a circumferential defect using chisels and burs with saline irrigation. Resection can be restricted to the inter-root area which measures ~4 mm in height and 3 mm in width (36–38) or extended to create a horizontal circumferential defect up to 5 mm below the fornix of the furcation (13, 39, 40) (Fig. 18.2). Some protocols elect to extract the first mandibular premolar followed by amputation of the first molar level with the crest of the surgically reduced alveolar bone (30). After resection, bone and cementum are removed, and notches are created to mark the lowest point of exposed roots in order to make a distinction between the old and new bone levels during evaluation.
2. *Regenerative treatment*: In the acute defect model, lesions are immediately treated with test agents. In the chronic defect model, the root surface is exposed to the oral environment in order to minimize spontaneous tissue regeneration

(30). Regenerative surgery can be performed after 4–6 months (30, 41) of healing in combination with scaling and root planing via open flap surgery. Regenerative molecules are then applied around the defect area and flaps are closed.

3.2.4. Post-surgical Management

1. *Pain and infection control:* Animals should be monitored until alert and active, and adequate homeostasis is achieved. While the animal is semi-conscious, it should be monitored every 15 min and then monitored every hour until fully conscious. Once conscious, the animal must be monitored twice daily until fully recovered from the surgical procedure. Recovery should be carried out in a quiet, temperature-controlled, designated recovery area separate from the normal cage. Administration of carprofen every 12–24 h and/or buprenorphine every 8–12 h can be carried out for post-surgery pain relief (*see Note 17*). Antibiotics, such as penicillin G benzathine (50,000 U/kg) should be administered subcutaneously on the day of surgery and every 5 days for up to 2 weeks.
2. *Oral hygiene maintenance:* For hygiene control, the treatment sites are to be cleaned with 0.1–0.2% chlorhexidine gel for 3 weeks following surgery. After the 3-week period, canines will receive a supragingival debridement and prophylaxis every 2 weeks. Every effort should be made to maintain optimal oral hygiene for each animal. Acepromazine (0.03–0.25 mg/kg i.m., i.v., or s.c.) may be used as a tranquilizer prior to oral hygiene measures, if necessary.
3. *Diet and miscellaneous:* The animals are to be given a soft chow diet for up to 2 weeks following all surgical procedures to minimize trauma to the periodontium, until dry chow can be tolerated (*see Note 12*). Sutures must be removed between 10 and 14 days post-surgery, under anesthesia if necessary. Surgical records should be kept in the animal housing area for 3–7 days following anesthesia and/or surgery.

3.2.5. Sacrifice and Harvesting

1. *Sacrifice:* At study endpoints, animals can be sacrificed and tissues harvested for analysis. Experimental endpoints are determined based on previous studies, published literature, and/or may be based on the biological material properties themselves. If the molecule of interest acts early in the healing process, endpoints should be selected accordingly in order to capture important healing events involving the compound of interest. Euthanasia methods, such as barbiturate overdose, should be selected according to institutional guidelines. A secondary method should be employed in order to confirm animal death prior to tissue collection. Harvested samples should be immediately fixed to prevent degradation or damage occurring to the tissues.
2. *Harvesting tissue:* For rat jawbone harvesting, a round diamond disk saw are used to cut hard tissues and scissors are used with soft tissues. The entire jaw can be harvested in whole. For canine jaw bone harvesting, a coping saw can be used to cut hard tissues. Each tooth segment can be sectioned by using a diamond saw.

3.3. Histological Sample Preparation

3.3.1. Fixation

1. 10% Formalin: 24–48 h, then replaced by 70% ethanol.
2. Ethanol: At least 72 h.

3.3.2. Decalcification—Use 10% EDTA at 4°C for 2–4 weeks. Alternatively 10% acetic acid, 4% formaldehyde, 0.85% NaCl for 2–3 weeks.

3.3.3. Dehydration and Infiltration Process of Paraffin-Embedded Specimens (In Tissue Processor/Autotechnicon)

1. Ethanol series [70, 80, 95% (twice) and 100% (three times)] for 1 h in each dilution.
2. Xylene for 0.5–1 h (twice).
3. Wax (paraffin) bath for 1.5 h (twice).

3.3.4. Casting for Paraffin-Embedded Specimens

1. Pour melted wax (use paraffin oven) to enclose specimen on the metal plate.

3.3.5. Sectioning for Paraffin-Embedded Specimens

1. Use a microtome to achieve a final thickness of 4–8 µm for glass slide mounting.

3.3.6. Dehydration and Infiltration of Epoxy Resin Specimens

1. Ethanol series [70, 80, 90, and 100% (twice)] for 1 h in each dilution.
2. Acetone for 2 h.
3. Propylene oxide for 15 min (twice).
4. 1:1 EPON 812/propylene oxide for 1.5 h.
5. 2:1 EPON 812/propylene oxide overnight.
6. EPON 812 for 30 min to 2 h at room temperature.
7. EPON 812 for 16–24 h in 55–65°C.

3.3.7. Dehydration and Infiltration of PMMA Specimens

1. Ethanol series [70, 80, 90, 95, and 100% (twice)] for 12 h in each dilution at 4°C.
2. Xylene for 12 h at 4°C (twice).
3. 1:1 PMMA/xylene for 24 h at 4°C.
4. PMMA and 0.5% hardener for 24 h at 4°C.
5. PMMA infiltrate for 2–3 days at –20°C.

3.3.8. Sectioning for Plastic-Embedded Specimens

1. Use EXAKT cutting device or ISOMETRIC diamond saw blade to achieve the final thickness of around 200 μm .
2. Mount on the plastic slide using methyl cyanoacrylate glue.
3. Polishing: Use EXAKT grinding system with sandpaper from 800 to 2,400 grits to achieve a final thickness of around 50–100 μm .

3.3.9. Hematoxylin and Eosin Stain (For Paraffin-Embedded Specimens)

1. Immerse in xylene (Fisher) for 3–5 min (*see* Table 18.2).
2. Rinse in gradient concentration of ethanol (70, 80, 90, and 100%).
3. Gill's hematoxylin 1 for 2 min.
4. Rinse with water.
5. Eosin–phloxine B for 1 min.
6. Rinse in gradient concentration of ethanol (100, 90, 80, and 70%).
7. Xylene for at least 3 min.

3.3.10. Methylene Blue Staining (For Epoxy Resin- or PMMA-Embedded Specimens)

1. Remove embedding material (*see* Table 18.2) (42).
2. 0.1% Formic acid (Fisher) for 1–2 min.
3. Rinse with water.
4. 95% Ethanol for 2 min.
5. 100% Ethanol for 2 min.
6. Xylene-float on the surface for 6–8 min.
7. 100, 95, and 70% Ethanol for 2 min in each.
8. Toluidine blue solution at 37°C for 4–5 min or Sanderson'sTM Rapid Bone Stain for 10–12 min.
9. Rinse in distilled water.
10. Counter stain by acid fuchsin (Sigma).

3.3.11. Modified Goldner's Masson Staining

1. Remove the embedding material on the surface of the specimen (43) (*see* Table 18.2).
2. Alkaline alcohol solution (90 mL of 80% ethanol, 10 mL of 25% ammonia) for 1 h.
3. Rinse with water for 15 min.

4. Weigert's iron hematoxylin for 1 h.
5. Rinse with water for 10 min.
6. Rinse in distilled water for 5 min.
7. Ponceau–fuchsin–azophloxine solution (5–10 mL Ponceau–fuchsin, 2 mL azophloxine, and 88 mL of 0.2% acetic acid) for 5 min.
8. Rinse in 1% acetic acid for 15 s.
9. Phosphomolybdic acid–orange G solution for 20 min.
10. Rinse in 1% acetic acid for 15 s.
11. Light green solution for 5 min.
12. Rinse in 1% acetic acid for 15 s (three times).
13. Rinse in distilled water.

3.3.12. von Kossa Staining

1. Remove the embedding material on the surface of the specimen (27) (for undecalcified sections only, *see* Table 18.2).
2. Silver nitrate exposed to strong light for 30–60 min.
3. Rinse with water (three times).
4. Sodium thiosulfate for 5 min.
5. Rinse with water.
6. Counter stain by safranin O.

3.3.13. Immunohistochemical Stains

1. Remove the embedding material on the surface of specimen (8, 27) (*see* Table 18.2).
2. 10 mM Sodium citrate for 10 min at 90–95°C. Cool to room temperature, rinse with 50 mM Tris-HCl twice.
3. Block with 3% H₂O₂ in 50 mM Tris-HCl for 5 min.
4. Wash and rinse with water.
5. 1:50~1:1,000 dilution of primary antibody in 50 mM Tris-HCl and 1% bovine serum for 10 min to 2 h (varies with manufacturer's instructions).
6. Wash and rinse with water.
7. 1:50~1:1,000 dilution of secondary antibody for 10 min to 2 h (varies with manufacturer's instructions).
8. Wash and rinse with water.
9. 3,3'-Diaminobenzidine (DAB) staining solution for 3–5 min.

10. Counter stain by Gill's hematoxylin 1.
11. Mount by permount and cover with glass cover.

3.4. Result Evaluation

If the study involves evaluation of the safety of new bioactive molecules, the following examinations are suggested:

1. Clinical observation – Changes in body weight, local swelling, edema, and/or inflammation of the surgical area.
2. Hematology – Complete blood cell and platelet counts, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, and red blood cell distribution width measurements.
3. Clinical chemical parameters (from serum) – Albumin, alkaline phosphatase, alanine transaminase, amylase, aspartate transaminase, bilirubin, calcium, cholesterol, creatine kinase, creatinine, glucose, phosphorus, total bilirubin, total protein, and globulin levels.
4. Major organ examinations – Surgical site and local tissues, salivary glands, local lymph node drainage, brain, heart, lung, liver, spleen, kidney, and gonads (testes or ovaries).

3.5. Descriptive Histology/Histomorphometry

3.5.1. Descriptive Histology—For studies involving tissue regeneration, histological images provide basic evidence of the effect of molecules of interest on cells and tissues. Therefore, it is indispensable to clearly identify and display the histological results. Every detail must be described in order to understand and explain the findings, especially to identify tissues affected, including but not limited to the affected tissue boundaries, location, collagen fiber alignment, cell characteristics, and tissue maturation.

3.5.2. Histomorphometry of the Rat Model—The following criteria can be measured for histomorphometry analysis:

1. Length of new cementum
2. Length of new bone
3. Length of alveolar bone fill (8)

3.5.3. Histomorphometry of Canine Periodontal Regeneration Model—The following criteria can be measured for histomorphometry analysis (44, 45):

1. Area of new bone.
2. Length of new cementum (*see Note 18*).
3. Length of complete new attachment apparatus (CNAA).
4. Percentage of defect filled with new bone and new cementum.

5. Length of crestal bone resorption/formation.
6. Length of apical migration of functional epithelium.
7. New bone formation can be further measured (46) with the following criteria: bone surface/perimeter, bone volume/area, tissue volume/area, core volume/area, osteoid surface/perimeter, eroded surface/perimeter, mineralizing surface/perimeter, osteoblast surface/perimeter, and osteoclast surface/perimeter.

3.6. Microcomputed Tomography(μ CT)

Conventional histological examination or histomorphometry provides direct representations of alveolar bone levels and histopathological phenomena with high resolution. In addition, these methods can analyze soft tissues, lamellar or woven bone quality, or external circumferential lamellae. However, this destructive method has a limitation to quantify whole regenerated periodontal tissue. In the case of analytical quantification and anatomical visualization of the mineralized tissue structures, micro-CT imaging is currently highlighted in medicine with a series of polychromatic radiographic images with micrometer-level voxel size. Micro-CT can provide a more accurate general profile and more detailed information in 3D structure (47).

3.6.1. Advantages of Micro-CT

1. Provides 3D assessments of whole and regenerated osseous tissues.
2. Provides volumetric mineralized tissue measurement (*see Note 19*).
3. Bone density can be calculated.
4. Degree of mineralization can be calculated.

3.6.2. Disadvantages of Micro-CT

1. Difficulty in identifying borders of newly mineralized tissue from old mineralized tissue.
2. Limited information of soft tissue, such as range of junctional epithelium.
3. Limited information regarding cellular level histology.
4. If the specimen contains metallic material, image scattering may occur around it.

4. Notes

1. Notes and records of the entire surgical and post-surgical processes are critical in ensuring proper animal care and lead to greater understanding of experimental results. Records should include, but are not limited to, animal identification, date/time of surgery, pre-surgical body weight, dose and route of anesthesia, period of anesthesia, surgical treatment, length of surgery, recovery period, surgical outcome, manner of disposition, and USDA identification number (canines only). Documentation of 5–7 days post-operative monitoring should be kept for all rodent procedures. Documentation of canines' post-anesthesia and post-

surgical medical records should be kept for 3 years following the completion of the study.

2. *Animal selection considerations.* Generally, in vivo experiments should move from small to large animals. Small animal models cost less and are generally easier to house, access, and work with. Upon completion and confirmation of small animal testing, large animal models can be used as they are physiologically more similar to humans, and experimental results will better translate to humans for potential clinical research. Specifically relating to oral surgical models, canines were historically chosen for their similarity in histological arrangement of canine periodontium and den-to-gingival attachment to that of humans.
3. *Strain, age, and gender.* The strain and age of the animal should be considered in order to achieve experimental objectives and for comparison to other studies. In young rats (7), periodontal regeneration is complete after 1 month, while geriatric animals at 18 months of age show a delayed healing capacity (48). It is therefore crucial to select the appropriate time point(s) to determine the therapeutic efficacy of a bioactive molecule. It should also be considered that as animals age, they will have decreased life expectancies and may develop systemic complications that are not externally visible. The specific animal strain is critical to understanding the animals' response to the surgical procedure and treatment. Gender can also affect the results, especially if the animals are osteoporotic, and viral transduction, for example, may be different according to the gender (49). Previous experiments, vendor information, and published literature should be used in determining the details of the selected animal model.
4. Treatment delivery vehicle should be chosen based on previous experiments and published literature. Delivery material should be biocompatible and easy to handle within the confines of the experiment. It is necessary to test the release kinetics of test articles from the candidate delivery material prior to animal experiment initiation.
5. *Surgical instrumentation.* The periosteal elevator will be used for flap elevation, the Gracey curette for root planing, and the bur in handpiece with engine and chisel for defect creation.
6. *Animal preparation for rodent surgery.* Following anesthesia, ophthalmic ointment (e.g., Puralube®) must be applied to the eyes of the animal receiving anesthetic to prevent drying. Incision sites must be cleared of hair if the incision is >1 cm, using clippers, a razor, or depilatory agent. Hair removal should be performed in a separate location so as to avoid surgical area contamination. Skin must then be disinfected with three alternating scrubs of iodophor (e.g., Betadine®) and warm, sterile saline, water, or 70% ethanol (ethanol is less desirable), scrubbing in an outward and spiral direction.
7. All instruments should be cleaned and sterilized (e.g., autoclaved) prior to surgery. Disinfection/sterilization of multiple sets of instruments should be carried out for multiple surgeries. Following use, instruments should be

thoroughly cleaned before sterilization. Hot-bead sterilization is a fast, dry method to prevent cross-contamination between animals during surgery. Alternative sterilization methods may incorporate the use of Sporocidin[®], chlorine dioxide, or Cidex[®] immersion followed by a sterile water or saline rinse. Aseptic techniques and sterile environments are critical to animal survival and positive experimental results. Effective drug dosage may vary from animal to animal according to body weight, metabolism, and age. There are no exact calculations to relate effective dose between animal and humans. Dosage can be determined by previous study results, published literature, and veterinary guidelines.

8. *Rat anesthetics and analgesics.* A combination of ketamine (i.p., 40-90 mg/kg) and xylazine (i.p., 5-10 mg/kg) can be used as a general, injectable anesthesia for oral procedures. To prolong anesthesia, supplement with one-third dose of ketamine only. IP injections should be performed using a 20-27-gauge needle that is inserted into the lower left abdominal quadrant with the animal in a head-down position. Buprenorphine (subcutaneous, 0.01-0.05 mg/kg) can be used for 8-12 h for post-operative pain relief or 5 mg/kg ketoprofen (subcutaneous) may be selected for 24-h pain management. Anesthesia depth is typically monitored by the loss of response to external stimuli, such as a limb pinch.
9. In some cases, the parotid gland duct (Stenson's) can be involved, causing post-surgical buccal swelling. This swelling can affect tissue regeneration as it produces mechanical pressure to the surgical area. Swelling can be eliminated by surgical removal of the parotid gland in those rare cases.
10. During bone and cementum removal, it is difficult to irrigate with saline due to the small defect size, thin bone, and cementum. Special care should be taken not to generate heat damage at the surgical site, as it prevents a normal healing process.
11. Antibiotics may be added to the drinking water in order to reduce the incidence of infection following oral surgery. Ampicillin (268 mg/L) may be added to a 5-10% dextrose solution. Colored water bottles should be used with light-sensitive antibiotics.
12. Animals should be mobile and feed freely following surgical recovery. Animal recovery time will vary with the type of anesthesia and dose of anesthesia, and may also vary between animals of similar sex, size, body mass, and genetic background. During the recovery process, all animals should be housed individually.
13. If viral vectors are used, each virus will have a specific shedding period for which it can be considered contagious, and contact should be limited during this period. For example, adenovirus should be considered biohazardous for at least 72 h following application/inoculation.
14. *Animal preparation for surgery – canine.* Preparation must be carried out in a room separate from the actual operating areas. Following pre-anesthesia

administration, prior to induction, ophthalmic ointment must be applied to the eyes of the animal to prevent drying. Hair removal from the surgical site will be performed in a separate location so as not to contaminate the surgical area. Sites will then be disinfected with three alternating scrubs of iodophor (e.g., Betadine®) and warm, sterile saline, or water, scrubbing in an outward spiral direction. Cephalic catheter can be used for ease of i.v administration of fluids and drugs.

15. *Canine anesthetics.* BAG (i.v. combination of 0.01-0.02 mg/kg buprenorphine, 0.03-0.05 mg/kg acepromazine, and 0.01 mg/kg glycopyrrolate) can be used as a sedative, 3-6 mg/kg of propofol (i.v.) can be given to effect for anesthesia induction/intubation, and 1-2% isoflurane (inhalation) can be used to maintain general anesthesia during surgery. Atropine may be utilized as needed during canine surgery for the treatment of bradycardia while the animal is under general anesthesia. Local pain relief may be relieved by 1-2 mg/kg bupivacaine. Anesthesia depth can be monitored by loss of blink reflexes, jaw tone (tense = inadequate anesthesia), heart rate, and respiratory rate (60-80 beats per minute, 10-12 breaths per minute with anesthesia).
16. If blood loss does occur during surgery, three times the estimated volume of blood loss should be administered via intravenous fluids.
17. *Canine analgesics.* Carprofen (2–4.4 mg/kg, subcutaneous) and/or buprenorphine (0.01–0.02 mg/kg, subcutaneous) can be used for post-operative pain relief.
18. The process of regeneration must be considered when determining time of sacrifice. Canine cementum formation (50, 51) on root planed surfaces occurs after approximately 3 weeks. Considerable amounts of new cementum and new bone filling the furcations may form after 6 weeks. The use of bioactive molecules may accelerate the healing process; thus preliminary studies should be completed with specific test articles to determine ideal evaluation time points in pilot studies.
19. The 3D volume is calculated by measuring the area in a slice-by-slice manner. After interpolating the region of interest and generating 3D constructs, bone parameters can be calculated. The measurements can be affected by the frame selected. Thus, natural anatomy such as distal and mesial roots can be used as landmarks for choosing the frame which can produce reliable and reproducible results (47).

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Fig. 18.1.

Illustration of rat periodontal defect model. (Reproduced under permission from Pellegrini et al. (6).

Fig. 18.2.

Illustration of dog periodontal defect model. NMF, new medical formulations; NB, new bone; PDL, periodontal ligament; NC, new cementum; and D, dentin. (Reproduced under permission from Pellegrini et al. (6).

Table 18.1

Materials for periodontal/craniofacial repair (reproduced with permission from Anusaksathien et al. (52))

Biomaterial	Trade name
<i>Allografts</i>	
Calcified freeze-dried bone, decalcified freeze-dried bone	Grafton [®] , Lifenet [®] , Musculoskeletal transplant Foundation [®]
<i>Xenografts</i>	
Bovine mineral matrix, bovine-derived HA	Bio-Oss [®] , OsteoGraf [®] , Pep-Gen P-15 [®]
<i>Alloplasts</i>	
Hydroxyapatite dense HA, porous HA, resorbable HA	
Tricalcium phosphate (TCP), calcium phosphate cement	Synthograft [®] , α -BSM [®]
Hard tissue replacement polymers	Bioplant [®]
Bioactive glass (SiO ₂ , CaO, Na ₂ O, P ₂ O ₅)	PerioGlas [®] , BioGran [®]
Coral-derived calcium carbonate	Biocoral [®]
Polymers and collagens	
Collagen	Helistat [®] , Collacote [®] , Colla-Tec [®] , Gelfoam [®]
Poly(lactide- <i>co</i> -polyglycolide), PLGA	
Methylcellulose	
Hyaluronic acid ester	Hy [®]
Chitosan	
<i>Growth factor</i>	
Platelet-derived growth factor (PDGF)	
Enamel matrix derivative (EMD)	Emdogain [®]
Bone morphogenetic protein (BMP)	
Fibroblast growth factor	
Insulin-like growth factor	
<i>Growth factor + scaffolding material</i>	
Collagen+BMP-2	Infuse [®]
β -TCP+PDGF-BB	GEM21S [™]

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Table 18.2
Staining methods and typical staining characteristics for each cellular component or tissue

Objects	Hematoxylin and eosin stain	Methylene blue stain	Modified Goldner's Masson stain	von Kossa stain	Immunohistochemical stain
Nuclei	Blue/black	Blue/black	Blue-gray		
Cytoplasm	Pink	Pink			
Bone	Pink	Pink			
Osteoid	Pink	Pink	Orange-red	Red	
Fibers	Deep pink/red	Deep pink/red		Red	
Mineralized tissue			Green	Gold/Brown	
Cartilage			Purple		
Antigens					Brown
Background					Pink/blue