HHS Public Access

Author manuscript

Periodontol 2000. Author manuscript; available in PMC 2015 November 16.

Published in final edited form as:

Periodontol 2000. 2015 June ; 68(1): 66–82. doi:10.1111/prd.12052.

Animal models for periodontal regeneration and peri-implant responses

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Translational application of experimental knowledge to the clinical setting is the ultimate goal of biomedical research. The reductionist strategies of the past are being rapidly replaced with a more holistic approach to the design, rationale and execution of research activities. Increasingly, the justification for basic research must be based on relevance and applicability to a clinical problem. However, as a prerequisite to clinical studies, mechanistic models to explain causality of observations and treatment outcomes are required. Although some work can be accomplished using *in-vitro* systems, ultimately animal systems are required for testing the safety and efficacy of data acquired before transfer to humans. Animal testing, as a prerequisite to human clinical exposure, is performed in a range of species, from laboratory mice to larger animals (such as dogs or nonhuman primates). In dental research, the animal species used is dependent largely on the research question or on the disease model. In general, smaller species are more convenient for disease-associated research, whilst larger animals are more appropriate in studies targeting tissue healing. Compared with smaller species, the anatomy of larger animals bears a closer resemblance to human dento-alveolar architecture. The recent emergence of new biologic approaches for the treatment of oral diseases, based on growth factors, active molecules and stem cells, has created a new need for animal research in dentistry in order to apply such techniques to humans. This review focuses on the animal models available for the study of regeneration in periodontal research and implantology; the advantages and disadvantages of each model; the interpretation of data acquired; and the future perspectives of animal research, with a discussion of possible nonanimal alternatives.

General overview

Animal experimentation dates back to the earliest days of research. A recent PubMed survey yielded more than 2000 peer-reviewed articles in which various animal models had been used for periodontal or peri-implant wound healing and regeneration studies. Within the context of periodontal research, animal models were first used to determine the relationship between infection and the host response in an effort to understand the disease process (53). Considerable data have been generated in animals to define the etiology and pathogenesis of periodontal and peri-implant diseases. Periodontal disease and, by analogy, peri-implant disease, are complex infections that result in a tissue-degrading inflammatory response (180). Many *in-vitro* systems provide strong evidence elucidating the cellular response to bacteria and bacterial products and consequently define the signaling pathways that are involved in this dynamic disease. However, it is impossible to explore the complex pathogenesis of periodontitis or peri-implantitis using only reductionist *in-vitro* methods. For example, multispecies biofilm models can be used for the characterization of host-

parasite interactions (91). On the other hand, these techniques can never fully reproduce the complexity of *in-situ* biology. There will always be a need for *in-vivo* models (46). Adding to the disease complexity is the recognition that the inflammatory response of the host is not linear (86, 181). For instance, within the context of periodontal tissues and biofilm-induced infection of the dentition, the number of microbial species associated with disease increase as a function of inflammation whilst further stimulating the inflammatory response (92). This observation provides strong evidence of direct and complex impacts of bacteria on the host response. Homeostasis in the oral cavity does not appear to be regulated in the same way as in the rest of the digestive tract, in which the progression of disease generally results in a reduced microflora, as seen in the gut (9, 24).

In addition to the disease process, healing of the periodontal and peri-implant tissues can be studied in animals. Regeneration, after periodontal surgery, in response to various biologic materials with potential for tissue engineering is a continuous process involving various tissue compartments, including epithelia, connective tissues and the alveolar bone (14). The same principles apply to peri-implant healing. Given the complexity of the biology, animal models are necessary and serve as the standard for successful translation of regenerative materials and dental implants to the clinical setting.

Multiple animal species, including mice, rabbits, hamsters, dogs, pigs and nonhuman primates, have been used to address these questions. In some animals, periodontal disease may occur spontaneously, whereas in others, it needs to be induced experimentally. A major drawback of smaller animals is the limited similarity of their dentition to human dentition. However, small-animal models generate substantial and relevant data on the interactions between soft and hard tissue, especially during inflammation, and hence periodontal inflammatory models, as well as peri-implant pathologies, can be simulated and tested in small animals. Another major advantage of small animals is that the potential mechanisms of systemic inflammation and its impact on periodontal healing processes can be studied *in vivo* using genetically produced transgenic and knockout animals (53). Finally, small animals are cheaper, and the use of small animals potentially eliminates the need to use larger species before human trials. To this end, mouse, rat and rabbit represent well-defined systems, and other small animals (e.g. hamsters) are also useful.

The dental anatomy of larger species of animals resembles human dentoalveolar architecture more closely than does that of smaller animals. They allow a more direct interpretation of the data and translation of the knowledge to clinical settings. Several large-animal species, such as nonhuman primates, dogs, sheep and miniature pigs, have been traditionally used in periodontal research. Nonhuman primates have oral structures and teeth similar to those of humans and have naturally occurring dental plaque, calculus, oral microbial pathogens and periodontal disease (125, 154). Histologically, the structure of their periodontium is similar to that of humans. During inflammatory processes, connective tissues are infiltrated by plasma cells, lymphocytes and neutrophils (126). Orthodontic brackets and ligatures can be used to accelerate plaque accumulation and periodontal disease (107). The drawback of using nonhuman primates is that they are susceptible to certain infections, such as tuberculosis (154), and ethical considerations and regulations have to be adhered to, to avoid any trafficking of protected species (167). To minimize cost and the aforementioned

concerns, excellent models have been generated using nonprimates such as miniature pigs. The major limitation of larger animals is that they are expensive and require specialized breeding and maintenance facilities, which limits their use to only specialized centers equipped with appropriate staff. Nonetheless, they are the *in situ* model systems phylogenetically closest to humans and hence highly applicable for testing materials destined for investigation in human clinical trials (125).

Rodents

Mice, rats and hamsters provide several advantages for evaluation of microbial and host responses. Rodents have only one incisor and three molars in each quadrant. Studies using rodents have shown disease development, after placement of ligatures in the gingival sulcus around the molar teeth, through an increase of biofilm accumulation (39). This model may not reproduce all aspects of human periodontitis initiation and progression; however, mice are useful animal models for using to understand host–parasite interactions. Young mice can develop periodontitis caused by their own microbiota if their ability to control their indigenous bacteria is compromised by genetic defects in their phagocytes, although the administration of antibiotics prevents the development of the disease (11). During periodontal studies, rodents are generally infected orally with select human pathogens, in an attempt to document the virulence of these species. These approaches are dependent on the use of genetically manipulated strains of rodents to focus on individual components of the host response and thereby to describe the roles of these components in the disease process (4). More recently, different investigators have used gingival tissue inoculated with chemicals, microorganisms or their products to elicit periodontal disease (117). In rats, periodontitis appears to be an infectious process. Inoculations or injections of various human periodontal pathogens can cause periodontal lesions (89). The breeding and housing costs of rodents are relatively low, making it possible to carry out studies with sufficient numbers of animals for statistical analysis. The knockout or transgenic models overexpressing various genes in mice and rats are readily available for use in studying the involvement of specific genes in periodontal and peri-implant diseases, and such approaches are being incorporated into research of regeneration. Furthermore, rodents are suitable models, not only for studies involving teeth, but also for studies investigating the dynamics of soft- and hard-tissue interactions relevant to oral inflammatory conditions. Thus, studies on rodents will continue in order to identify the pathways of regulation of inflammation, resolution and healing processes in periodontal tissues as a first step in translating the basic science into *in-vivo* models.

The mouse calvaria model, although not a true periodontal or peri-implant system, has potential use in a wide variety of disease applications relevant to dental research (53). Experiments utilizing the mouse calvaria model can provide valuable data on the efficacy of many compounds, including those with regenerative potential. Similar strategies have been used in other species, such as rats or rabbits. In this model, soft- and hard-tissue healing can easily be studied and the role of various molecules in the regulation of bone turnover and regeneration can be investigated. Knockout or overexpression of genes of interest during healing can be assessed. Classical inflammatory events are induced following a stimulus injection directly into the connective tissue overlying the calvarial bone, including the rapid

expression of proinflammatory cytokines within a few hours and the recruitment of neutrophilic granulocytes within 24 hours (54). Bone resorption can be induced within 3–5 days, depending on the size of the stimulus (103). The mouse calvaria model is useful in studying bone turnover and the sequence of inflammation, destruction and repair relevant to large-animal models.

Mice are the smallest species used widely in dental studies. There are several advantages of the mouse model. They are extremely cost-effective compared with larger animals. Antibodies against mouse antigens are almost as widely available as human panels, which allow researchers to study a wide range of immunohistochemical end-points in mice. In recent years, mice have also become the animal of choice for knockout studies, with an extensive genetic array for different backgrounds. The mouse genome has been sequenced; therefore, the specific roles of genes in the regulation of disease processes, inflammation and regeneration, including periodontal disease, can be conveniently studied in mice. Mono-infection, virulence or mixed-disease models can be studied in mice (108, 201, 204). Periodontal disease and inflammatory processes can be induced in diabetic mice or in mice with a certain predisposition to cardiovascular diseases (6, 100, 106, 108, 124, 200, 204). The disadvantages of mice are their body size, as well as the dimensions of their teeth. Although various studies have successfully shown that ligatures can be tied around murine teeth to induce periodontal disease, this is an extremely difficult task (57, 102). The oral-gavage approach is an alternative (45, 53, 115). In this technique, an external pathogen is grown in broth and introduced into the oral cavity of the mice by gavage. Periodontal disease usually develops within 7–10 days. The mechanism of action probably involves conversion of the commensal microflora in mice to a pathogenic biofilm during this process, and tissue responses can be studied. As the dietary habits of rodents are substantially different from those of humans, oral biofilm formation in mice and rats after ligature or gavage (58) requires further and detailed investigation in order to interpret the findings appropriately.

Rats have also been extensively used in periodontal disease studies. Various approaches have been used to induce periodontal inflammation in rats. Currently, two techniques are widely employed. The first requires administration of bacterial lipopolysaccharide to the gingiva of the rat, creating an acute and severe periodontal pathology (29). This model has the advantages of generating a rapid disease process and not requiring growth of bacteria or extensive manual handling of the dental structures. Therefore, it is used especially for testing the efficacy of various agents to resolve inflammation (16, 17, 88, 122, 143, 176, 182). The disadvantages are that the model is an acute inflammatory response to the endotoxin of the bacteria, the infection is not generated by the bacteria and the host response probably represents specific pathways in certain cell types. With those limitations in mind it should be noted that valuable information on the action of various compounds, as well as testing the actions of the lipopolysaccharide and other confounding factors in periodontal destruction *in vivo*, has been generated using this model. Conversely, the placement of ligatures around teeth to initiate periodontal tissue loss leads to the accumulation of plaque and ulceration of the sulcular epithelium, facilitating invasion of connective tissue. Ligature-induced periodontal disease in rodents does not require human pathogens. However, alveolar bone

loss in the ligature model is dependent on bacteria, as the placement of ligatures does not induce significant gingival inflammation or periodontal bone loss in gnotobiotic rats (53, 144). Thus, these models could continue to be useful for addressing specific questions, as *in-vivo* models.

Rabbit model

Rabbits also represent a good “small animal model” for studying the impact of inflammation on periodontal wound healing and regeneration. In addition to being small enough to conduct a reproducible periodontal inflammation study around teeth, a major advantage of rabbits is that the ligature alone does not induce destruction of soft or hard tissue. Hence, it can be used to test the specific impact of periodontal pathogens of human origin, in contrast to rats and mice, in which ligature placement induces periodontal disease by conversion of the commensal flora into a pathogenic flora in the oral cavity. Another advantage of the rabbit model is its use in nondental inflammation models, such as cardiovascular diseases. Rabbit aorta is commonly used to investigate lipid deposition and vascular changes. Although rabbits do not develop spontaneous atherosclerosis, they are useful because they are highly responsive to cholesterol manipulation and develop lesions in a relatively short period of time (38). The high-cholesterol-diet rabbit model has been widely used for inducing experimental atherosclerosis. In normolipidemic rabbits, atherosclerotic lesions can develop as a result of repeated or continuous intimal injury by an aortic polyethylene catheter, balloon angioplasty or nitrogen exposure (145). Therefore, many studies have used the rabbit model with a high-cholesterol diet, arterial wall injury, or, most commonly, a combination of both. The observed lesions resemble, at least in part, those seen in human plaques, regarding the inflammatory component and the vascular smooth-muscle-cell proliferation. The rabbit model has largely been used to study the influence of lipid lowering (through diet or statins) on plaque formation and stabilization. Those studies have contributed to unveiling the mechanisms by which lipid lowering reduces macrophage accumulation and other aspects of atheroma inflammation. Rabbits with inflammation have a greater number of vascular lesions than do rabbits without inflammation. This model could represent a novel approach to the study of inflammation-associated atherosclerosis (111). A model for plaque rupture has been also developed in rabbits through the combination of aggressive vascular injury and a hyperlipidemic diet (159). This technique accurately quantifies fibrotic and lipid components of atherosclerosis in the model and may permit the serial analysis of therapeutic strategies on atherosclerotic plaque stabilization. Thus, rabbits are an excellent model for studying the inflammatory basis of periodontal diseases and healing patterns.

Rabbits represent a relevant model in which the physiology and the pathology of periodontal tissues resemble those of humans with respect to pro- and anti-inflammatory mechanisms. Previous work, by our group, has shown that a predictable and reproducible periodontitis can be generated in rabbits by using silk ligatures together with topical application of the periodontitis-specific microorganism, *Porphyromonas gingivalis* (65, 66, 77). In this model, silk ligatures are tied around the second premolars of the mandibles of rabbits (Fig. 1). Ligature alone does not result in periodontal tissue destruction. Application of *P. gingivalis*, every other day for 6 weeks, reproducibly generates periodontal disease in rabbits, with

clinical, radiographic and histologic patterns very similar to those observed in humans during development of periodontal disease. Stopping the application of the pathogen at the end of this period does not result in spontaneous resolution of inflammation or in tissue healing. The efficacies of various compounds have been tested using this model (64-66, 68, 69). Whilst the preventive impact of therapeutics can be investigated by the simultaneous administration of bacteria and the therapeutic agent during the entire 6-week study period, treatment of the periodontal disease can also be studied by stopping the administration of bacteria and testing the ability of the compound of interest to resolve the inflammation and the impact on bone (68). Bone coupling has been shown as an outcome of resolution of periodontal inflammation and regeneration in this model. Transgenic rabbits are also available and have been used to study periodontal and atherosclerotic diseases. The major disadvantage of rabbit models is the limited availability of anti-rabbit antibodies; however, this issue is being rapidly resolved as a result of the increasing applicability of rabbit models in biomedical research.

In addition to these studies, rabbits are readily used for studying the integration of dental implants into bone. Most of these techniques involve the long bones of rabbits. These studies have been designed not only to test the integration of new materials and surface characteristics introduced in implant designs (10, 18, 19, 25, 26, 30, 31, 59, 80, 90, 148, 166, 170-172, 196, 199), but also to analyze the confounding factors of bone healing, such as bisphosphonates, taking advantage of bone turnover patterns in rabbits (28, 177). The impact of various regenerative procedures around implants has been tested in rabbit models (19, 25, 48, 78, 81, 123, 147, 175, 183, 198). Some of these models have incorporated the use of the rabbit calvaria (87, 175). Very limited work has utilized the alveolar bone in rabbits for periodontal regeneration or dental implant studies (1, 23, 162).

Dog model

Dogs are among the most well-established models to study naturally occurring gingivitis and periodontitis (128). The Beagle dog is commonly used because of its size and its extremely cooperative nature; however, many studies have also used mongrel dogs (167). Periodontal tissues and the size of the teeth in dogs are, in general, similar to those in humans. However, dogs lack lateral jaw movements and occlusal contacts at the premolars. The subgingival bacterial species are predominantly anaerobic gram-negative cocci and rods (41, 61). The severity of periodontal disease in dogs increases with age and can result in natural tooth loss. Periodontitis follows gingivitis; periodontal pockets develop with a dense cellular infiltrate consisting mainly of plasma cells and lymphocytes. The osteoclastic resorption of alveolar bone results in deep, narrow lesions extending vertically around a single root, leaving the interdental space intact (60). Conversely, the extent and localization of periodontal lesions may not be always predictable in dogs (63, 167). Animal care regulations, including daily companionship, exercise, space and maintenance, make use of dogs increasingly less desirable and convenient in periodontal disease and inflammation studies (125). Nevertheless, the dog model is still extensively used in wound healing, dental implant and regeneration studies (193).

The dog as an experimental model in periodontal regeneration is not new. For decades, dog studies have been considered as the major *in-vivo* testing system for new regenerative devices or techniques. Some of the earliest evidence on the efficacy of guided tissue regeneration came from dog studies (120). The dog model was also used to assess the impact of biomechanical relationships in the oral cavity within the context of regenerative procedures (138). Dogs served as excellent models for studying the impact of periodontal inflammation and plaque control on regeneration and new attachment formation (73, 138, 139). These studies, and later work, demonstrated the regenerative potential of the periodontium, providing breakthrough evidence that regeneration around previously diseased root surfaces was possible provided that periodontal ligament cells could selectively repopulate onto the roots, whilst other cell types did not have the capacity to regenerate new “connective tissue” attachment, confirming the earlier observations in humans (13, 114, 120, 121, 192, 194, 195). Following these critical observations, dogs have been extensively used in various landmark studies in periodontal regeneration and eventually in implant dentistry.

During the early 1980s and 1990s, identification of the optimal materials and techniques for regeneration in periodontal therapy relied heavily on dogs as the experimental model of choice. Dogs are affordable compared with monkey models, are easy to use and their availability and size are practical. Various graft materials have been tested as bone substitutes (101, 136, 168, 169), as has the efficacy of root surface conditioning in inducing periodontal regeneration (13, 15, 118). Guided tissue regeneration and membranes have been studied in dogs, and a substantial number of publications demonstrate the efficacy and biocompatibility of expanded-polytetrafluoroethylene, synthetic and organic resorbable membranes (44, 62, 109, 110, 119, 135, 173, 174). Comparative studies of resorbable and nonresorbable membranes provided a wide selection of membrane configurations for use in guided tissue regeneration (22). The major contribution of dog studies in regenerative periodontal medicine has been the histological demonstration that guided tissue regeneration is achievable in various defect types, such as furcations and infrabony resorption sites (21). Similarly to studies in humans, dog models have helped in gaining an understanding of the limitations of regenerative approaches with regard to defect type (e.g. Class III furcations) and size, as well as of membrane-associated properties (e.g. resorption kinetics and type), providing a basis for the combined use of bone substitutes and barrier membranes (35, 110, 140). Such studies further demonstrated that defect characteristics and space are key elements for success in periodontal regeneration (188), and the combination of root-surface treatments with barrier membranes did not enhance the outcomes of guided tissue regeneration (40). Dogs were also used for other periodontal-wound healing models. In one study, no difference was observed when barrier membranes or free connective tissue grafts were used to cover the recession defects (190).

Growth factors and their regenerative potentials in periodontal defects have also been tested in dogs. Lynch and colleagues demonstrated that platelet-derived growth factor and insulin-like growth factor enhanced periodontal regeneration, resulting in significant gain in new bone formation and attachment (105). A single administration of platelet-derived growth factor plus insulin-like growth factor stimulated new-attachment formation in dogs (49).

Platelet-derived growth factor was found to stimulate fibroblast proliferation during early wound healing, and barrier membranes did not significantly change the outcome (186); however, insulin-like growth factor, fibroblast growth factor and transforming growth factor-beta1 did not enhance bone formation, fibroblast production and collagen density (156). Some studies demonstrated that the response around all teeth in dogs was different and that time was critical for a significant regeneration. For example, in a study in which Class III furcations were treated with membranes alone or with platelet-derived growth factor, the second premolar teeth showed significantly better regeneration compared with the fourth premolars, and addition of platelet-derived growth factor enhanced new bone formation by up to 87% (32, 127). Such limitations, were further explored in dogs (7, 12), defect dimensions were analyzed (165) and some novel concepts, such as cell seeding, were tested (37). These investigations support the use of combined technologies in extreme defects, such as one-wall intrabony destructions (178, 206) or vertical augmentation (160), and in some new approaches, such as the application of recombinant human growth/differentiation factor-5 with poly(lactic-co-glycolic acid) constructs (96).

Specific roles of various extracellular matrix proteins, such as type I collagen, fibronectin, secreted protein acidic and rich in cysteine, vitronectin and bone sialoprotein, during regenerative healing was identified using the dog model (113). Fibronectin and vitronectin were found to be involved during early healing events (71), but their application did not enhance membrane efficacy (95). Flap designs and tensile strength were analyzed histologically in dogs (191). Collectively, these reports confirm that the regenerative potential of periodontal tissues depends on regulation by early biological events and includes an orchestrated series of events in which certain biologically active molecules (e.g. growth factors and extracellular matrix proteins) could play important roles at various stages of healing.

Enamel matrix proteins have been tested in periodontal regeneration in dogs, and the efficacy of such preparations in this respect has been demonstrated (3, 5, 47, 55, 70, 74, 76, 134, 146, 189). These studies are significant in the light of the wide use of enamel matrix proteins in several periodontal and peri-implant applications and have shown that new alveolar bone, as well as new cementum formation, can be facilitated through their use whilst preventing the apical migration of the epithelium (5, 146). An interesting study also demonstrated that while nicotine impaired membrane-induced regeneration, the efficacy of enamel matrix proteins was not affected (134). Another valuable contribution was the observation that regenerative techniques using enamel matrix derivatives not only resulted in enhanced hard-tissue formation, but also increased gingival tissue thickness (3).

Regeneration of bone around dental implants has also been tested in the dog model since the early 1990s. Various surface preparations, as well as designs of implants, were tested with the concomitant use of barrier membranes, and the results suggested that the use of these materials facilitated peri-implant regeneration (202), even when the implants were placed in fresh extraction sockets (27), providing some of the early evidence for the feasibility of immediate implant placement. However, not all implant designs and membranes tested in dogs were found to be successful in regenerating bone around implants. For example, a hydrolyzable polyester material (polyhydroxybutyrate-hydroxyvalerate reinforced with

polyglactin 910 fibers) not only did not enhance new bone formation, it actually resulted in an increased inflammatory reaction with significantly less bone formation compared with the control side, which was treated without any barrier membrane (52). Likewise, the prototypes of some membrane designs, such as one with two collagen layers and an internal polylactide layer for lateral ridge augmentation, did not show any benefit in dogs (185). Nevertheless, the results of many dog studies on regeneration around implants have proven to be beneficial in providing data applicable for use in humans (8, 203), whilst demonstrating the efficacy of such methods (184). These studies further justified the need for the use of carefully designed studies in animal models before clinical application in humans.

In addition to clinical efficacy, factors complicating guided bone regeneration outcomes following the experimental induction of peri-implantitis in dogs have also been studied. For example, the lack of oral hygiene and membrane exposure during healing were found to impair bone regeneration and integration (51, 56, 158). Likewise, inflammation has been shown to be a complicating factor during implant loading (93). Implant stability was tested in peri-implantitis models in dogs (50, 157), and novel technologies, such as laser treatment, were studied (36), with significant findings suggesting that the inflammatory process and the resulting alveolar bone destruction in peri-implantitis in dogs are similar to those in humans and can be successfully treated. These studies also initiated a debate on defect types and material composition, as well as on the surface characteristics of implants (130, 132). For example, dehiscence-type defects have been proposed to have a higher chance for predictable restoration of tissues. In addition, infection and inflammation were shown to result in a challenge for resolution and periodontal/peri-implant regeneration. In several studies, resolution of inflammation was shown to lead to healing and “re-osseointegration” on the surfaces of previously contaminated implants after removal of accumulated plaque (82, 112, 131), whilst connective tissue, to some extent, was also found between the implants and the bone (129). Titanium-reinforced membranes were found to be predictable in protecting the space for blood-clot stabilization around implants and resulted in supracrestal bone formation, even without the addition of bone grafts (83). This approach was tested around implants in which the threads were left exposed, and vertical bone growth was observed when nonresorbable membranes were used as space maintainers (142).

Taken together, the dog is still being used as an experimental model, although its use has substantially decreased for several ethical reasons as well as because of the introduction of other small-animal models or, in many cases, replacement with alternative models such as the miniature pig. Nevertheless, it continues to serve as an excellent model for identification of the mechanism underlying the tissue response to regenerative approaches in periodontology and implantology.

Miniature pigs

The miniature pig model has emerged as a good alternative to the dog model (Fig. 2). Varieties of miniature pig have been extensively used in biomedical research, studies of artificial organs and dermal wound-healing models since the 1950s (42, 137, 155, 179, 197). These animals are similar to humans in gross anatomy and physiology and have economic

advantages over other large species. Some of the miniature pigs develop human diseases, such as diabetes, through similar processes and therefore represent an excellent model for systemic diseases (98). A recent and excellent review has described the dental characteristics of miniature pigs in detail (187). The periodontium of miniature pigs shows many similarities to that of humans. The depth of the gingival sulcus is 2–3 mm with a long junctional epithelium. Natural gingivitis can be observed at 6 months of age. The pattern of disease progression follows the same stages as that in humans: gingival swelling, plaque accumulation, calculus formation and bleeding on probing. Histologically, these clinical features are accompanied by inflammatory cell infiltration and vasodilatation. As of 16 months of age, miniature pigs may develop advanced periodontitis, with pocket depths up to 5 mm and alveolar bone resorption. In an experimental periodontitis model in miniature pigs, in which ligatures were placed and human periodontopathogens were administered, periodontitis developed in 4 weeks, with increased periodontal destruction by 8 weeks and no further progression until 20 weeks (187). Removal of the ligatures did not result in complete restoration of the periodontal destruction, with remaining substantial tissue loss supporting the suitability of the model. Almost the earliest nondisease use of miniature pigs in periodontal research was for the creation of mucogingival defects (84, 85). In this model, facial gingiva was excised from primary incisors and resulted in recession by 3 months. This model was not further explored in follow-up studies but certainly would be of use. Miniature pigs have also been tested for suitability in experimental peri-implantitis studies. Silk ligatures were placed around dental implants for 45 days and disease was assessed by measuring increases in pocket depth, loss of attachment and gingival inflammation (72). During peri-implantitis in miniature pigs, the microbiota changed from primarily gram-positive facultative organisms to gram-negative obligate anaerobes, and, similarly to human disease, bone loss was evident and implant threads became exposed.

Over the last two decades, miniature pigs have been heavily used for testing the regenerative potential of various compounds and technologies. In a series of studies, the osseointegration of dental implants was tested in grafted bone, demonstrating that iliac crest grafts were significantly better compared with hydroxyapatite (150-153). Lack of proper bone formation in response to alloplastic materials was further confirmed in miniature pigs (116). In addition to testing bone substitutes, miniature pigs were used for guided bone regeneration procedures around implants (161). Combined use of autogenous grafts and membranes demonstrated that guided bone regeneration was predictable and that osseointegration, bone regeneration and disease processes could be accurately assessed in the miniature pig (20, 149). Histologic examinations demonstrated that bone repair involved a programmed maturation and that bone grafts did not enhance membrane-induced regeneration. Histomorphometric analysis showed that autologous bone grafts had the best osteoconductive properties during the initial healing period, with 39% of newly formed bone inside the membrane-covered defects at 4 weeks of healing, and that the regenerative process was similar to that in humans (20). We have developed a miniature pig model for immediate implant placement and loading and tested a novel biodegradable polymeric material combined with a light/chemical hardening technology for bone grafts. Implant stability and function; microcomputed tomography, histologic and histomorphometric analyses of bone–implant contact; and the characteristics of newly formed bone around

implants and extraction sockets have been tested. The results contribute to our knowledge on the importance of primary stability in the success of immediate implants and immediate loading (67).

As novel technologies developed, many of the new devices were tested in miniature pigs. Enamel matrix proteins have been tested in miniature pigs; the results suggest that application of enamel matrix proteins alters the type of periodontal connective tissue interface (33). In another study, autogenous periodontal cell grafts, with or without the application of enamel matrix derivative, were tested on the implant–connective tissue interface. The study demonstrated that the implants, treated with periodontal cells but not with enamel matrix derivatives, demonstrated good bone contact with some epithelium; however, the best results were obtained for the group in which the implants were treated with periodontal cells and enamel matrix derivatives – significant bone contact was observed without any evidence of the epithelium (34). The miniature pig model was also used to test the efficacy of platelet concentrate in bone regeneration; no supportive role was found for platelet concentrate in the regenerative actions of autografts or bone substitutes (79). In one study, the impact of platelet-rich plasma was tested on flap strength at various postsurgical time points in a miniature pig model. Platelet-rich plasma did not seem to contribute to greater flap strength and did not enhance wound healing (141). One of the recent applications of miniature pigs in periodontal regeneration has been the testing of growth factors and their regenerative capacity. The regenerative capacity of recombinant human bone morphogenetic protein 7 was tested following the extraction of immature primary incisors and the total removal of periodontal ligament and cementum. Recombinant human bone morphogenetic protein 7 improved both the survival and the eruption of replanted teeth (164). A recent bone-augmentation study compared recombinant human bone morphogenetic protein 7 with autogenous bone particles using maxillary sinus augmentation techniques in minipigs (99). The results of the study clearly indicated that minipigs are useful models in various bone-regeneration procedures and produce relevant data that can be easily translated into standard clinical applications in humans. Overall, whilst a series of studies on miniature pigs supported human data, it also opened new avenues of potential therapeutic applications.

An interesting model involved the replantation of cultured cells from the periodontium of miniature pigs, demonstrating the formation of new cementum and new bone (97). This study was significant in showing that regeneration in the periodontium was determined by the availability of precursor cells capable of forming calcified tissues. Recent studies further suggest that not only is the miniature pig an excellent large-animal model for regenerative studies, it also harbors a good source for pluripotent cells, which may be harvested for use in regeneration (75). Thus, tissue-engineering studies extensively utilize miniature pigs. For example, a new population of stem cells isolated from the root apical papilla of human teeth was transplanted into miniature pigs to generate a root–periodontal complex capable of supporting a porcelain crown and normal tooth function (43, 163), demonstrating that complete periodontal regeneration was possible (104). These studies support the feasibility of using stem-cell-mediated tissue engineering to treat periodontal defects. The most recent research has tested how the regeneration of multiple periodontal tissues is coordinated in miniature pigs. The hybrid tooth–bone constructs combined tooth-bud-cell-seeded scaffolds

with autologous iliac crest bone-marrow-derived stem-cell-seeded scaffolds that were then transplanted back into surgically created mandibular defects in the same miniature pigs. The constructs were harvested after 12 and 20 weeks of growth; the tooth-like structures consisted of dentin, enamel, pulp, cementum and periodontal ligament and were surrounded by regenerated alveolar bone (205). This strategy was also tested for coordinated autologous reconstruction of tooth and mandible (2). Stem cells were shown to promote tooth regeneration in autogenic cell transplantation on various scaffolds (94). Combining the mesenchymal stem cells and platelet-rich plasma incorporated into a fluorohydroxyapatite scaffold in cylindrical defects in the edentulous mandibular ridge of miniature pigs demonstrated the formation of new vital bone (133).

Conclusion

Animal experimentation in dental research is necessary and will stay relevant. Animal models are often superior to *in-vitro* or clinical studies in addressing mechanistic questions and serve as an essential link between hypotheses and human patients. There is no single animal model that represents all aspects of human disease, tissue architecture and healing and aging processes. However, human studies cannot always be coupled with harvesting the tissues and body parts necessary for microscopic analyses, which are necessary for defining the biologic impact of the regenerative methods and materials. Therefore, animal studies are critical for establishing cause-and-effect relationships and, just as importantly, for initial tests of principles in the development of new regenerative devices and advanced therapeutics. In selecting the animal model, the goal should not be to define the model which is most similar to humans but rather to describe how various models can be used to provide insights into the mechanisms of periodontal diseases, wound healing around implants, tissue architecture and responses, and whether a given model is suitable for studying a specific hypothesis. As animals are vulnerable, models should be carefully planned and justified. Power calculations in such studies are crucial to target a sample size large enough to generate statistically useful data, whilst at the same time small enough to prevent the unnecessary use of animals.

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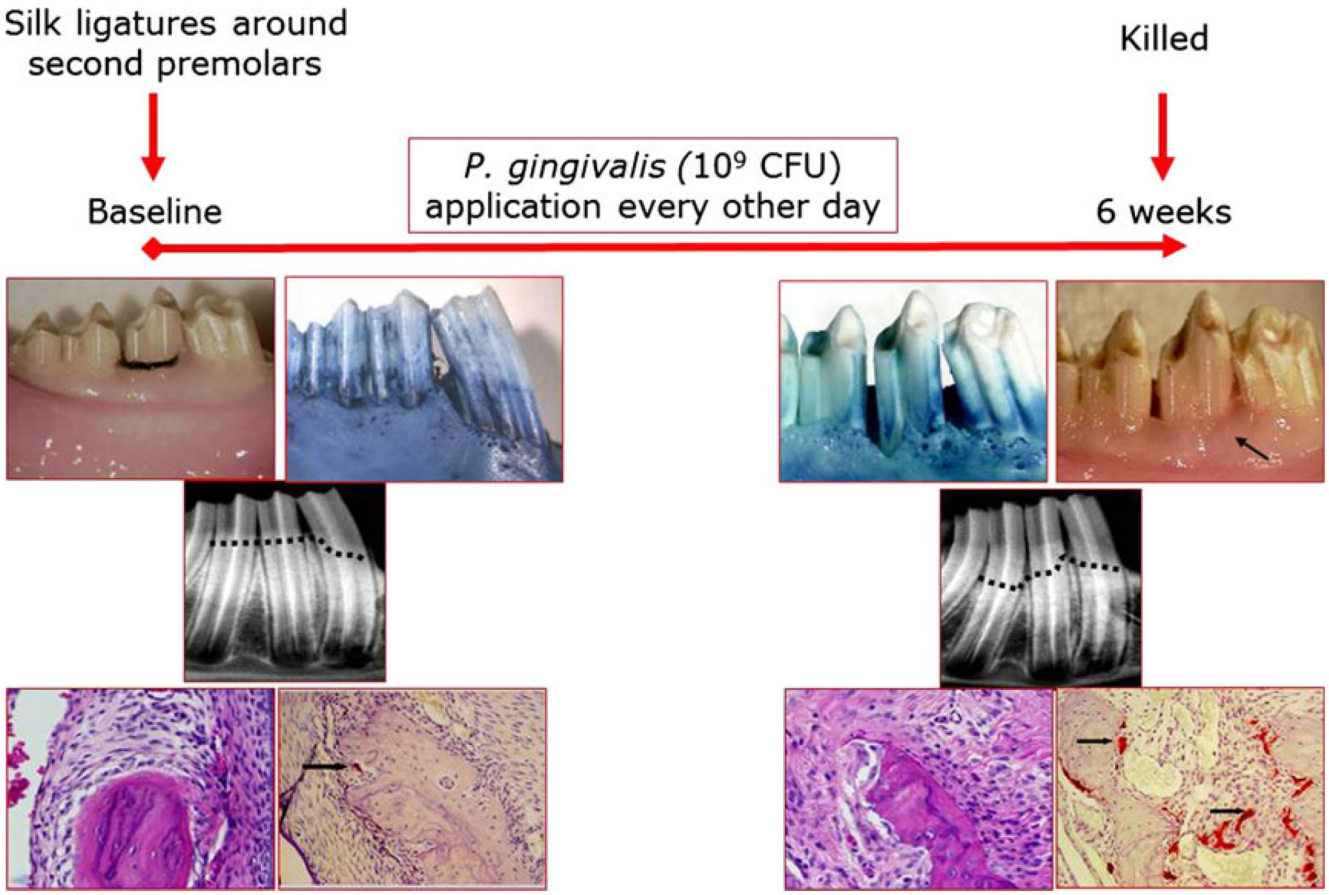

Fig. 1.

Rabbit as a model for periodontal disease. In New Zealand White rabbit, periodontal disease can be established by placing silk ligatures around the second premolars. The rabbit oral microbiota does not result in periodontal disease development upon accumulation around the ligatures. A pathogenic bacterium (e.g. *Porphyromonas gingivalis*) can be applied throughout the study (6 weeks). Clinical, radiographic and histologic evidence demonstrate clear periodontal tissue destruction, similar to the disease process in humans. Established periodontal disease in rabbits is not reversible when left untreated (68). CFU, colony-forming units.

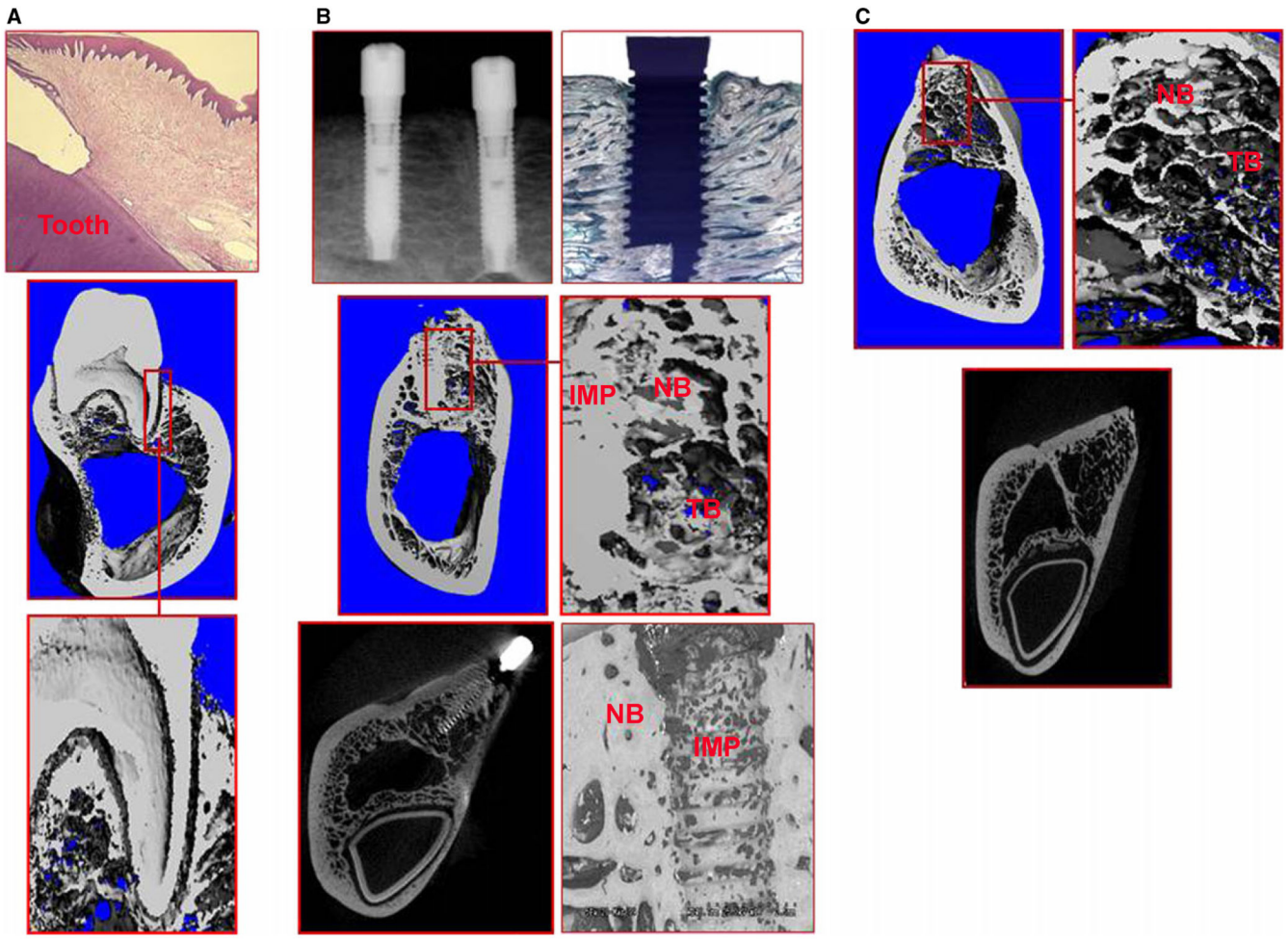

Fig. 2.

The miniature pig as a model for periodontal and peri-implant studies. (A) Miniature pigs can be used in periodontal disease studies as a large animal model as a result of the resemblance of their dentition with human dentition. Clinical measurements can be accompanied by radiographic measurements and by three-dimensional analysis using micro-computed tomography. (B) Miniature pigs can be used as a model for implantology research. Histologic, clinical and radiographic, as well as electron microscopic, analyses can be made. IMP, implant; NB, new bone; TB, trabecular bone. (C) Miniature pigs can also be a good model for socket preservation studies in which trabecular bone and new bone formation can be studied using radiographic and qualitative methods.