IN VIVO ANIMAL MODELS IN PERIODONTAL RESEARCH – FOCUS ON RODENTS

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Summary

Firkova, E. I. & Ts. Chaprazov, 2021. *In vivo* animal models in periodontal research – focus on rodents. *Bulg. J. Vet. Med.,* **24**, No 2, 167−175.

Periodontal research has developed very fast in the last two decades. Although at this stage of science a lot of genetic and molecular-based trials are performed in order to elucidate the complex etiology, pathophysiology, biofilm-host interactions and responses on genetic and cellular level*, in vivo* animal models are still used. In many ways, *in vivo* experiments are superior to *in vitro* tests when the dynamics of the immune-inflammatory nature of the periodontal disease and peri-implantitis and the specific healing of soft and hard tissues is concerned. Screening the efficacy, mechanisms of action and application of different biomaterials requires *in vivo* experiments, before the data translation to clinical settings. A number of small animals like rodents and large species like dogs and nonhuman primates are involved in periodontal research. As live creatures are used, the design of the studies must be well defined, with regard to the type of the animals, most suitable for the tested hypothesis, observation period, sample size, study power, critical size defects, and specific testing sites.

Key words: animal models, biomaterials, periodontal research, regeneration

INTRODUCTION

Regenerative periodontology and implant dentistry has developed very fast in the last two decades, with a variety of new biomaterials and approaches being introduced, which claim sufficient regenerative and healing potential. *In vivo* animal models are necessary and still stay relevant in modern dental research. Although a lot of research is accomplished using *in vitro* systems, animal experiments are still the most important step and the essential link between the hypothesis and the human patients. Usually after a positive *in vitro* testing for a certain hypothesis, evaluation of proof-of-principle concepts, safety and unwanted reactions are performed in pre-clinical studies before proceeding to clinical testing.

Staying up-to-day with modern principles and requirements for proper design of

the scientific projects, this review aims at general to summarise and outline basic *in vivo* models, used for preclinical studies in periodontology and implant dentistry. It is also focused on experimental procedures and the surgical techniques used to test bone grafting biomaterials in rat model.

GENERAL OVERVIEW

Animal experiments date 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 regeneration and wound healing studies (Kantarci *et al.,* 2015). The first animal models were used to find out the relationship between periodontal infection and the host response (Hamp *et al.,* 1972; Hamp & Lindberg, 1977; Hugoson & Schmidt, 1978). Many studies were conducted to define the complex etiology and pathogenesis of periodontal diseases. Although there are successful and relevant *in vitro* models, they cannot explain in details, for example, the specific host-parasite interactions. *In vitro* methods can never fully reproduce the complexity of *in situ* biology. There are some topics that can be elucidated only in animal models, e.g. wound healing and regeneration process in the affected periodontal tissues. The regeneration after periodontal surgery is a slow, continuous process, involving epithelial cells, connective tissue, periodontal ligament and the alveolar bone. The effects of different biomaterials, used to substitute the bone loss or to enhance the healing process in soft tissues, or possible side effects, can be validated only through well designed and performed animal studies (Sculean *et al.,* 2015). Actually the European Medi-

cines Agency and the US Food and Drug Administration often require a series of targeted pre-clinical evaluations before human trials are started.

Knowing the complexity of periodontal biology, microbial biofilm-host interactions and principles of soft tissue healing and alveolar bone regeneration, animal models are necessary and serve as standard for successful translation of regenerative biomaterials to the clinical setting.

ANIMAL SPECIES USED IN PERIODONTAL AND BIOMECHANICAL RESEARCH

Evaluation of bioactivity, biocompatibility, toxicity, potential adverse reactions or side effects and efficacy of a biomaterial intended to enhance bone formation requires models, closer to humans. A preclinical *in vivo* model should be based on similarities between the processes studied in animals and humans in health and disease, so that the data could be extrapolated to the human population. The ideal *in vivo* model should be performed with the phylogenetic species with anatomic similarities, biochemical and physiological mechanisms closest to those in humans (Oz & Puleo, 2011).

Multiple animal species, including mice, rats, rabbits, dogs, pigs and nonhuman primates have been used for those purposes. The selection of animal species must be based mainly on the research question or on the disease model.

The dental anatomy of larger species – dogs, sheep, miniature pigs, nonhuman primates – resembles more closely the human dentoalveolar structures. Beagle dogs are among the most used models to study naturally occurring gingivitis and periodontitis and later – peri-mucositis

and peri-implantitis (Klinge *et al.,* 2005; Lin *et al.,* 2017). Histological and radiographic observations revealed in details the destructive changes in epithelial and connective tissue attachment and cementum and the patterns of alveolar bone resorption after ligature-induced periodontitis (Nyman *et al.,* 1986) and peri-implantitis. The studies with dogs have been considered also as the major *in vivo* testing system for new regenerative techniques or devices. Some of the earliest evidence for the efficacy of guided tissue regeneration came from dogs (Nyman *et al.*, 1980). During the 1980s and 1990s, dog studies were extremely popular for testing of graft materials, resorbable and nonresorbable membranes, root surface conditioners (Levy *et al.,* 1981; Fleischer *et al.,* 1988; Magnusson *et al.,* 1990; Pontoriero *et al.,* 1992). Later, growth factors like platelet-derived growth factor, insulin like growth factor, as well as various extracellular matrix proteins for stimulation of new attachment formation was tested first on dogs (Lynch *et al.,* 1991; Cho *et al.,* 1995; Iqbal & Bamaas, 2001). However, in recent years, the use of dog models has been substantially decreased for several reasons – ethical considerations; replacement with miniature pigs or small animal models.

Miniature pigs have been extensively used in biomedical research dealing with dermal wound healing and artificial organs. Some of pigs develop human diseases, such as diabetes, with the same pathophysiological processes involved and therefore, they are a very good model for studying systemic diseases in the field of periodontal medicine (Kalkwarf & Krejci, 1983; Van Dorp *et al.,* 2002). The advantages of using a miniature pig model for dental and orofacial research were presented in an excellent review by Wang

et al. (2007). Although the periodontium of those pigs shows many similarities to that of humans and therefore they are suitable models for different investigations, to our knowledge pigs are still not commonly used in Bulgaria.

The major limitations of using large animals are that they are expensive; require specialised breeding and maintenance facilities and highly qualified staff and laboratories. There is also an increased risk of post-operative complications and the possibility of losing test animals in the course of the experiment.

The use of small animals (rodents, rabbits) has several advantages. The animals are of small size, which means less space for housing, easier breeding, facilities, etc. They are cheap which allows to include large number of animals for collecting sufficient data for analyses. Small animals are cost-effective also because they are easier to handle – the surgery procedures are easier and post-surgery recovery is faster; less complications can be expected compared to large animals. Small animals are more resistant to infections which reduces the risk of losing experimental animals during the course of the experiment.

Rodents are widely used animals in periodontal research. Rodents are suitable models for investigation of the dynamics of soft- and hard tissue interactions, different pathways of regulation of inflammation, signal molecules, bone turnover and regeneration, etc. They provide several advantages for evaluation of microbial and host responses. Rodents have 1 incisor and 3 molars in each quadrant. After placement of a silk ligature around a tooth and plaque accumulation, the development of periodontal disease can be evaluated. Actually rodents are used mainly to study the host-parasite interactions

(Graves *et al.,* 2008, 2012; de Molon *et al.,* 2014). Rats are often infected with human periodontopathogens in order to document their virulence factors (Klausen, 1991). Induced periodontal disease is subjected to detailed microbiological and immunological studies. Antibodies against mice antigens are available almost as human panels, which allows performing different immunohistochemical investigations. The mouse genome has been sequenced, therefore the specific role of different genes in the regulation of inflammatory and healing process can be assessed. The mouse calvaria model is well-known. The experiments can provide data on the efficacy of many molecules and biomaterials, used for periodontal regeneration (Lindhe *et al.,* 1993; Stavropoulos *et al.,* 2003, Silva *et al.,* 2017). Mice are convenient models to study the evolution of inflammatory and immune events. After injection of microorganisms, fast expression of proinflammatory cytokines and recruitment of neutrophil granulocytes is observed within 24 hours (Graves *et al.,* 2008). Bone resorption can be detected as soon as 3 to 5 days, depending on the dose of the initiating stimulus (Li *et al.,* 2002).

Rabbits are also good small-sized animals to study the impact of inflammation on periodontal and peri-implant wound healing and regeneration. A ligature, fixed around teeth or implants, does not induce by itself a periodontal inflammation. This fact allows the researchers to define clearly the specific effects of periodontal pathogens. In contrast, a ligature placed around mice and rats' teeth induces periodontal inflammation by conversion of their own commensal flora into a pathogenic one. Hasturk *et al.* (2006) were able to induce a predictable and reproducible periodontitis in rabbits by us-

ing silk ligatures together with topical application of *Porphyromonas gingivalis*. They observed clinical, radiographic and histologic signs of periodontitis, very similar to those in humans. Stopping the application of the pathogen at the end of the 6-week experiment did not result in spontaneous resolution of inflammation or healing. Using this model, a lot of compounds and their efficacy were tested with clinical implications of the results in humans (Hasturk *et al.,* 2007; 2009).

Rabbits are used also for studying osseointegration of implants. Most of these techniques involve the long bones as orthotopic testing sites (Indjova *et al.,* 2014). These studies test not only the integration of new materials and surface designs, but also the effect of confounding factors on bone healing and implant integration such as bisphosphonates (Chacon *et al.,* 2006).

EXPERIMENTAL MODELLING:
RATIONALE, RELEVANCE,
SIZE ISSUES

Planning an *in vivo* experiment for the needs of periodontal research, requries very careful consideration of all details, having in mind that live creatures are involved. The goals should be clearly established. Correct calculation of sample size and study power are very important, so that there is enough data for statistical analyses without unnecessary use of animals. For testing the efficacy, mechanisms of action, bone regeneration and soft tissue response and healing of different biomaterials is possible using 2 possible testing sites – heterotopic or orthotopic. The choice of test site is made on the specific hypothesis tested.

Heterotopic sites are within the soft tissues of the animal – intramuscular or

epimuscular, subcutaneous or intrafatty. Heterotopic sites are used when pharmacological and pharmacokinetic properties, as well as possible side effects and toxicity of different medications are evaluated (Schuh, 2008).

Orthotopic sites are used mainly when regenerative capacity of biomaterials is tested. Orthotopic sites can be intraosseous or periosseous or extra-skeletal. Intraosseous/periosseous site can be created in different locations – calvarium, mandible, long bones, sinus (Klinge & Johnson, 2012).

Selection of this model should be based on many factors (Stavropoulos *et al.,* 2015):

- Mechanism of action (whether the biomaterial has osteoconductive or osteoinductive properties);

- The physical properties of the material (small particles, block, gel, membrane);

- The intended clinical application (fill in or cover defects).

Probably the most useful pre-clinical model for bone regeneration materials is the intraosseous site model with a creation of so-called "critical size" defects. The latter are osseous defects that do not heal completely during the lifetime of the animal unless subjected to an intervention (Smitz & Hollinger, 1986). This model involves creation of cylindrical defects on the calvaria of small animals – rats, mice and rabbits. Different sizes of defects are used, depending on the species and the age. The critical size in mice is 3–5 mm, in rats: 5–8 mm and 15 mm in rabbits. Actually even a very small defect size will be critical, provided that the experiment is of short duration but reasonable interpretation of results can be made only if the defects have relevant dimensions, so that

the biomaterial can enhance itself the bone formation.

The procedure of creating the critical size defect in calvaria of rodents is standardised (Schmitz & Hollinger, 1986; Cooper *et al.,* 2010). Usually 2 symmetrical circular bicortical defects are created in the parietal and/or the occipital bone, so that the test and control sites can be observed being under the same conditions. Access to the calvarium is made by midline or lateral skin incisions, extending from the nasofrontal area to the occipital protuberance. After skin elevation and incision of the subcutaneous fascia, the bone is exposed by a blunt dissection. The anatomical characteristics of the calvarium allows easy creation of standardised, reproducible defects, usually made by trephine bur. The procedure must be done carefully, with respect to the animal following all surgical principles for good practice. Drilling of bone is performed by a trephine and traditionally 5 mm round defects are created. Care must be taken not to damage the underlying dura mater, blood vessels or cranial sinus. The bone disk must be removed very carefully, in order not to tear the subjacent cranial structures. If a large damage occurs in mice calvaria, it will result in protrusion of brain into the created bone defect, thus eliminating the test/experimental space. Moreover, the lack of dura continuity decreases the bone formation; when it is intact the defect show complete fill in immature rats (Mossaz & Kokich, 1981). After the procedure is performed, the periosteum, fascia and skin are repositioned and tightly sutured. It is important to note that dura has the ability to stimulate osteogenesis by expression of fibroblast growth factor-2 and transforming growth factor-β. In case of testing new biomaterials is reasonable to use barrier mem-

brane to limit the ingrowth of soft connective tissue and maintain a high concentation of locally produced osteogenic factors (Dahlin *et al.,* 1991).

The advantages of this "critical size" calvarial defects include the easy access and handling procedures; possibility for radiographic and histological analyses. A possible limitation is that there is no option for evaluating the biological response of the implanted biomaterial to physiological biomechanical loading.

The model is well defined, in means that it is known how long time it takes for the cortical defect to recover. It has been proved that a period of 4 to 8 weeks is necessary for the bone to regenerate to reach a plateau; beyond that period no significant bone formation can be expected (Gosain *et al.,* 2000).

Possible outcome variables in this model include (Stavropolous *et al.,* 2015):

- Extent of defect closure (length and area);
- Thickness of the osseous bridge;
- Relative and absolute volumes of tissue components (bone, connective tissue and biomaterial).

However, this approach probably will change in the near future. Some authors (Cooper *et al.,* 2010) suggest that the term "critical size defect" should be discontinued, because the outcomes (like quality of bone formation) can be more accurately evaluated by microcomputer tomography or other advanced techniques in live animals. Until such new approaches are widely accepted and approved as relevant for clinical interpretations, the critical size model is still the gold standard for bone healing strategies.

CONCLUSIONS

In vivo small animal models are essential for periodontal research. Testing the efficacy and specific mechanisms of action of different biomaterials used for periodontal regeneration and wound healing is necessary to translate the knowledge to humans. No animal model can completely recreate the specific characteristics of the human periodontal complex. That is why the design of such models must be carefully cleared in details, in terms of most suitable for the main goal (research question or disease model) species, observation period, sample size, study power, critical size defects, and specific testing sites. Post-operative care for the animals must be performed following the best practice.

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