

Methodological Consideration of Various Intraosseous and Heterotopic Bone Grafts Implantation in Animal Models

Weiguo Xu¹, Gerald Spilker¹ and Christian Weinand^{1,2*}

¹Department of Plastic and Reconstructive Surgery, University Hospital of Witten- Herdecke, Cologne Merheim, Germany

²Department of Plastic and Aesthetic Surgery, Hand Surgery, Burns, Dietrich-Bonhoeffer-Klinikum, University of Greifswald, Neubrandenburg, Germany

Abstract

Bone fracture healing is a complex process including inflammation, repair and remodeling. Bone grafts or substitute are widely accepted to treat impaired healing. Newly developed bone substitutes must undergo in vitro and in vivo testing before clinical application. All kinds of intraosseous and heterotopic implant models in small and/or large animals are used for different bone substitutes. Orthotopic implant at models is used for evaluation of graft materials. Based on the statistic of literatures, we find small animals should be used first before large animal as osseous defect models. Rabbit and rat are the commonly chosen animals, while femur and calvaria are the most implanted anatomic sites.

Critical size defect models are useful as bone defect model, but vary considerably between animals. Typical heterotopic ossification after implantation of bone substitute is found almost in all species of animals. No bone formation is found after subcutaneous implantation of bone granule grafts in small animal, but in large animal. In contrast, bone block grafts show a distinguished result of bone formation in small animals as well as in large animals. This article reviews currently animal bone defect models and anatomic implant site for bone graft, gives a recommendation for the future research.

Keywords: Implant design; *In vivo*; Orthopaedic; Tissue engineering

Bone Substitutes

Developments in material technology offer clinicians a variety of choices of bone substitutes for patients. Usually, bone graft materials are divided into four categories, e.g., autograft, allograft, alloplast and xenograft. The application of autologous bone grafting is gold standard in the therapy in bone defects by trauma, tumour resection, dental augmentation or osteonecrosis, although there are always potential risk and complications [1]. Allografts are taken from the same species with a different genotype, which have been treated by sterilization and antigenic procedures. There are several types of allografts: fresh or fresh frozen, freeze-dried and demineralised freeze-dried allograft [2]. Because of disease transmission, fresh allograft is not so frequently used [3]. On the basis of the development of material science, different kinds of alloplastic implant materials can be chosen by physicians as alternative. Most alloplastic implant materials are ceramics. They have several advantages: biocompatibility, non-antigenicity, lack of inflammatory response and resorbability, etc. [4]. Silicone implants have been most frequently used for chin and finger implants. In the last three decades, allograft based on hydroxyapatite and/or silicon has been widely used in orthopedic and dental areas, because of its osteoconductive capacity [5]. Xenografts are taken from another species, which have the same composition and identical morphology compared to that of human bone. But the surface antigens of xenograft may cause immunogenic response. Bovine bone was chosen as xenograft materials because of simple source and low cost [6].

Because the supply of autologous bone grafts is limited in clinic, allogenic and xenogenic bone substitutes are quickly developed. The ideal bone substitute should have characteristics of biocompatibility, osteoactivity and biodegradation [7,8]. Other desirable characteristic include mechanical stability, optimized scaffold and vascularisation, biologically appropriate chemistry and surface charge, unlimited availability and structural stability [9]. Biocompatibility means that bone graft materials do not release chemical toxic substances or cause immunological, allergic or other adverse reactions in the recipient

organism. Different methods in the ISO 10993-11 series of international norms are described to evaluate the biocompatibility of biomaterials [10].

Osteoactivity refers to the osteointegrative, osteoconductive, osteoinductive and osteogenic properties of an implant [11]. Osteointegration shows the capacity of bone graft direct binding to the surface of human bone without the growth of fibrous tissue at the bone-implant interface. Osteoconduction describes a bone substitute material supporting newly formed bone growth over its defined surface. Osteoinduction is the formation of new bone by active recruitment of mesenchymal stem cells from the surrounding tissue of the recipient. These stem cells differentiate into osteoblasts. This process is controlled by growth factors such as the bone morphogenetic proteins (BMP). Furthermore, BMP-incorporated bone implant material is applied in clinic [12]. Osteogenic properties refer to implants containing live cells, which contribute synthesis of new bone [13].

Implanted bone materials should be resorbed at a rate corresponding to new bone formation. Through the remodelling phase, biomaterials are replaced by new bone tissue [14]. Mechanical stability of the material provides adequate strength and mechanical stability according to the bone defect [15]. Vascularisation with sufficient blood supply is necessary for new bone formation and bone remodelling [16]. A high level of porosity and interconnection of pores in the material provides

***Corresponding author:** Christian Weinand, Department of Plastic and Aesthetic Surgery, Hand Surgery, Burns, Dietrich-Bonhoeffer-Klinikum, University of Greifswald, Neubrandenburg, Germany, Tel: +49 395 775 2802, E-mail: chwsco@yahoo.com

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a larger surface so that autologous proteins from the blood enter the nanopores and cover the entire inner surface [17,18].

Following the development of nanotechnology, some new bioactive nanohydroxylapatite are widely applied in bone tissue engineering, whose composition and crystal structure is very close to natural bone [19]. For example, a fully synthetic nanocrystalline hydroxyapatite embedded in a matrix of silica gel (NanoBone[®]) has showed new bone formation after five weeks while demonstrating degradation of materials [20]. Currently, there is no ideal material bone graft that can provide all requirements of bone defect in different defect site. Nevertheless, they provide certain advantages, such as good store, ready availability and the prevention of a further operation for patient. Furthermore, all kinds of bone substitutes have also been applied as cellular scaffolding system [21]. Cell-based bone graft substitute have shown significant osteogenic potential in animal segmental bone defect [22].

Experimental Animal Choices for Evaluation of Bone Substitutes

New developed bone replacement material needs strictly test *in vitro* and *in vivo* due to cytotoxicity and physical and chemical characterise before clinical applications for human [23]. Before beginning animal experiment, biomaterial should be at first tested by cell culture *in vitro* for cytotoxicity and cytocompatibility. Basic demand of a bone substitute material for *in vitro* cell culture is that the bone graft supports cell attachment, proliferation and migration [24]. A variety of experimental animal species including mouse [25,26], rat [27,28], rabbit [29,30], dog [31], pig [32] and goat [33] have been used to test bone grafts. Although most animal models are in quadrupeds and may not model the load bearing conditions evident with bipedal humans. But bone structure, composition and biology of large animal (i.e., dog, sheep, pig and goat) are very similar to that of human bone, which demonstrated a very similar bone healing process compared with human bone remodelling [34]. On one hand, the use of bone defects in large animal models is highly recommended to clearly evaluate the capacity of the tissue-engineered bone substitute for its final clinical application. On the other hand, using large animal models has two major limitations, for example, high costs and the care for the respective animals. Therefore, 38% of the studies in bone-healing research already preferred the rat as the experimental animal, following the next rabbit 19%, mouse 15%, sheep 11%, dog 9%, goat 4% and other 4% [35]. That means above 70% experiment animal species are small animal (rat, rabbit and mouse). Therefore, Le Guehennec and co-workers suggested that small animals should be a prerequisite before preclinical implantation of bone substitutes in large animals [36]. Small animal approach using rats or mouse allows easily studying a reasonable number of animals at different time points. In a dorsal skinfold chamber model of mouse, the inflammatory and angiogenic host tissue response to biomaterial can be on-line given for evaluation of biocompatibility and vascularisation *in vivo* [37]. Selection of animal species should be decided by purpose of research, clear questions and experimental conditions [38]. Otherwise, due to various anatomic, biochemical, and gene expression of experiment animals, the same animal model used in different species may show conflicting results in bone healing studies [39].

Intraosseous Critical/Noncritical Defect and Heterotopic Implant Model

Various animal models and implantation sites are applied to test bone substitutes by means of different evaluations of parameters [40]. There is the fundamental distinction between intraosseous and

heterotopic models for bone substitute materials. An intraosseous model allows analysis of osteoactivity and biodegradation of implanted materials in a bone environment [41]. The application of heterotopic implant model is gold standard for assessment of the biocompatibility as well as the osteogenic and osteoinductive potential of bone graft materials [42].

Using intraosseous model, bone graft materials are usually implanted in tibia [43], femur [44], ulna [45] and calvaria defect [46]. According to the size of the defect, in which the bone substitute will be implanted, they can be categorized as either critical or noncritical defects. A critical size defect is a segmental or drill whole defect with load or non-load mechanical stress, which does not heal spontaneously during the lifetime without pathological changes [47]. Frequently, segmental critical/noncritical size defect was chosen in animal models for testing functionality of bone substitute materials. Such defects are usually used to evaluate whether the bone graft material is in a position to bridge the defect [48]. The drill hole critical/noncritical defect is usually used to study a variety of bone defects and potential therapies designed to repair these bone defect, which is applied to test filling materials for bone defect [49].

By means of heterotopic implant model, the toxicity and carcinogenic effect of biomaterials could be also evaluated by direct contact with the subcutaneous and muscular tissue [50]. Cellularity and vascularity of the recipient bed are very important parameters for the osteoinductive capacity in the ectopic bone formation [51]. Subcutaneous and muscular implantation usually allows for an evaluation of the biocompatibility, angiogenesis, degradation and osteoinduction of the tested materials [52]. Using subcutaneous implant model in rat, dynamical changes are investigated after implantation of nanostructured hydroxyapatite until 12 days [53].

Lots of studies demonstrated that adipose tissue is a good source of adult stem cells with a multilineage differentiation potential, for example, bone, cartilage, skeletal muscle and other cell types [54]. Comparing to muscle tissue, adipose tissue is well-vascularized with a capillary density [55]. Hartman et al. reported that no significant difference existed between intramuscular and subcutaneous recipient sites after bone substitute implantation [56]. However, this method ultimately does not analyse the functionality of a bone substitute material, because subcutaneous tissue has different physiological structure and molecular biological reaction to implanted material compared to osseous environment [57]. Furthermore, new bone formation in heterotopic tissue does not provide information on the influence of biomechanical stress and dynamic load as intraosseous implantation [58].

In this review, we examined the literature relating to intraosseous and heterotopic model used large and small animal in the evaluation of bone substitute.

Bone Defect Model and Different Anatomical Sites for Implantation in Animals

Different species of experimental animals and defect models are applied in bone tissue engineering, but there have been little criteria for choice of animal and implantation site. Following rapid development of materials science, more and more new bone grafts are tested by means of experimental animals.

We have summarised literatures of the MEDLINE database during 1 January 2006 through 30 November 2015 for studies published about bone substitutes using bone defect models in different animals. In all

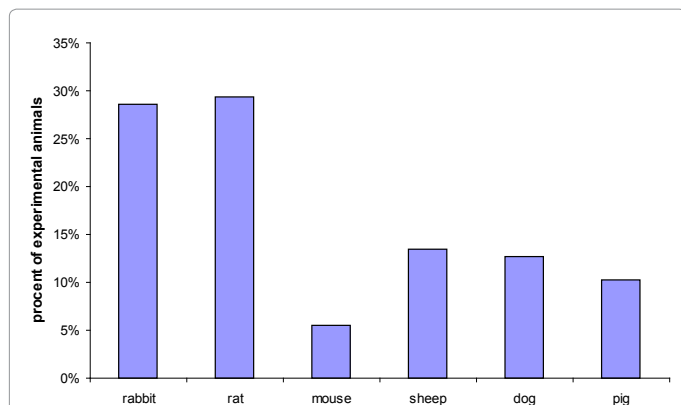


Figure 1: Quantitative statistic representing choices of various animals in bone defect models as derived from publications in pubmed over last fifteen years. Rat and rabbit have been the most widely used animals for such studies.

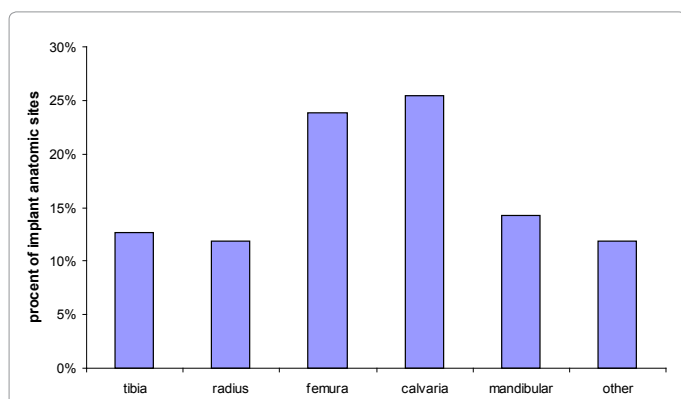


Figure 2: Distribution of anatomic sites for bone defect model, calvaria and femur are the most used region for bone graft implantation.

	tibia	radius	femur	clavaria (diameter)	mandibular
rabbit	8 [59]	15 [60]	15 [61]	20 [62]	15 × 10 [63]
rat	5 [64]	5 [65]	6 [66]	8 [47]	5 (diameter) [67]
mouse	NA	NA	5 [68]	4 [69]	4 (diameter) [70]
sheep	50 [71]	NA	25 [72]	22 [73]	35 (diameter) [74]
dog	NA	25 [75]	21 [76]	20 [47]	18 × 10 [77]
pig	20 [23]	NA	NA	9 [78]	50 [79]

Table 1: Summary of critical size defect (mm) in different anatomic site of animals; NA=not available.

	Bone graft	Anatomic site	Observation period	Bone formation	References
rabbit	PLGA/Type-I collagen+osteoblast	subcutaneous	4 weeks	+	82
	nanohydroxylapatite/polyamide block	intramuscle	4 weeks	+	83
rat	NanoBone granulate	subcutaneous	6 months	-	84
	Cortical bone cylinders	subcutaneous	4 weeks	+	85
mouse	Hydroxyapatite/demineralized bone matrix	intramuscle	8 weeks	+	86
	beta-tricalcium phosphate block	subcutaneous	6 weeks	+	87
sheep	Human demineralized bone matrix	intramuscle	8 weeks	+	88
	macroporous cement	subcutaneous	6 months	-	89
dog	calcium phosphate ceramics	intramuscle	6 months	+	90
	beta-tricalcium phosphate	subcutaneous	12 weeks	+	91
pig	HA/TCP biphasic ceramics	intramuscle	15 months	+	92
	nanocrystalline hydroxyapatite	subcutaneous	8 months	+	93
	tricalcium phosphate	intramuscle	12 weeks	+	94

Table 2: Typical examples of subcutaneous and intramuscular implantation in different animals.

experimental animals for bone defect models, 64% of all animals used were small animals. The small animals commonly chosen for bone defect models are rat (29%) and rabbit (29%). Mice are only used in about 6% of all cases, because of their difficult management (Figure 1). Among big animals, sheep, dog and pig dominate at almost the same ratio of 10% to 13% (Figure 1).

Bone grafts are made into different shapes in order to apply for different defect size and shape. Therefore, there is no established standard for anatomical implantation sites. However, independent from the animal employed in the experiments, long bones (49%) such as tibia (13%), radius (12%) and femur (24%) are commonly chosen as implantation region. Viateav states that the calvaria mimic the clinical bone defect environment best because of poor blood supply and presence of little bone marrow. Furthermore, calvarial bone is formed by intramembranous ossification rather than endochondral ossification [42]. Therefore, the calvarial defect model rate is as high as 25% of all defects used. Interestingly, bone grafts and implants currently applied in craniofacial surgery are increasing in number. In coherence with this tendency, mandibular defect model are found in literature in 14% of all cases. Other anatomic sites, such as the iliac, rib, ulna and spinal region, are used in about 12% of all cases (Figure 2).

Critical Size Defects of Animal Interaosseous Model in Different Anatomical Sites

Multiple critical size defects of animal models have been performed to mimic bone defect environment for evaluation of bone grafts. In the following table, critical size defect in different anatomic sites of animals are summarized (Table 1).

A critical defect size is associated with the size of the animal and anatomical site of bone. Reproducible critical size defect model is very important for study design. However, numerous studies *in vivo* demonstrated that sizes of critical defects in the same species vary strongly from study to study [59]. In addition, researchers must consider that many systemic factors such as age, sex, endocrine and pharmacological status of animal strain could affect results [60].

Heterotopic Model in Small and Large Animal Model

There are no standard criteria for intramuscular and subcutaneous implantations. Abdomen, back, limb and neck are popular regions for implant. In addition, the variety in different bone substitutes makes comparison difficult as each bone graft has specific weight, density and volume. The following table provides an overview of some publications about bone grafts implanted either subcutaneous or intramuscular (Table 2).

	Methods	Description	Reference
histomorphometry	histology	biocompatibility, osteoactivity, biodegradation	104
	histochemistry	biochemical property	105
	immunohistology	molecule identification	106
radiography	X-ray	densitometry	107
	micro-computed tomography	morphological characterization, quantitative assessment	108
	computed tomography	in situ evaluation	109
biomechanical test	torsional stiffness, shear stress, angle of fracture	mechanical strength	110
microscopy	scanning electron microscopy	morphologic characterization	111
	transmission electron microscopy	ultrastructural morphology	112
	energy dispersive X-ray spectroscopy	physicochemical characterization	113

Table 3: Methods selected in bone scaffold evaluation.

Herein, we give only some examples of heterotopic implant model. Many factors, for example chemical component and physical characters, size of implanted biomaterial and observation period can influence results. Interestingly, our previous work showed granule grafts did not induce bone formation in small animals, but block grafts induced bone formation in small animals. Yuan et al confirmed that material-induced bone formation depends on the animal species and implanted material [61]. Likewise, various bone graft combining growth factor or stem cells shows different osteogenic properties in various heterotopic models [62-65].

Mechanics of heterotopic ossification is not completely clarified. Kan et al demonstrated that morphogenetic protein (BMP) plays a crucial role in this case [66]. Heterotopic implantation induces hyperactivities of BMP receptors. Osteoinductive properties of BMP are reported first time by Urist et al [67,68]. Now recombinant BMPs (rBMPs) have been widely applied for treatment of spinal fusions, non-union fractures, craniomaxillofacial and periodontal bone defects, and bone/tooth implant augmentation [69], as they are readily available.

Methodical analyse of bone graft after implantation in animal

Bone grafts are made of a variety of components, structures, shapes and sizes; there are no established standard methods for analysing bone substitute *in vivo*. However, most often used methods are radiological evaluation using x-rays, mikro CT, or high resolution CT as well as histochemical or immunohistochemical analysis. The most often used methods are visualized in Table 3.

Discussion

Ideal bone substitutes should support osteogenesis, osteointegration, osteoconduction and osteoinduction and contain or propagate the ingrowth of osteoblast or osteoprogenitor stem cells. However, only autologous bone graft has, as mentioned above, these properties. During the past 30 years different bone grafts have been developed to provide structural stability in order to overcome the clinical drawbacks associated with bone defects.

Currently, all kinds of bone grafts including natural bovine-derived hydroxyapatite and hydroxyapatite or silicon based bone substitute are widely applied in clinic. As synthetic material mimics' composition and structure of extracellular bone matrix and can be customized for specific application without the limit of amount supply, this is especially interesting for clinical application in orthopedic surgery [70]. Using 3D based imaging of defect positions, biomodels can be produced ahead of operation in reconstructive surgery with availability of different size and

shape, thereby reducing operation time and patient trauma extremely and increasing the patient's safety [70-88]. All kinds of biomaterials combining BMP have shown successful effects in reconstructing long bones, spines and the facial skeleton defects [72-105].

Rational experimental design and careful selection of the animal model play a very important role in researching bone tissue engineering. No experiment model fulfils complete evaluation of new developed bone graft materials. A newly developed bone biomaterial should first be optimized *in vitro* [73,93-95,104]. After that, various parameters are further evaluated through small animal models [106-108]. In the next step, large animal models are used to test the practicability functionality of graft materials before clinical application [86,105,109-115]. For each specific question, a suitable animal model is needed. Each animal model has its own advantages and disadvantages. Intraosseous defect models are necessary to assess the possibility of clinical application for orthopaedic and craniofacial surgery. To evaluate biocompatibility, biodegradation and vascularisation, subcutaneous or intramuscular implantation models in mouse or rat are recommended [22,42,69,46,73,82,84-86,88,90,93,94,96,100-103]. But these models may not be suitable to answer questions of osteoinduction of granular bone grafts. A possible explanation for the difference between large and small animal in subcutaneous implantation of granule bone graft may be the increased micromovement in small animals [73,82,84-86,88,90,93,94,96,100]. Small animals show constant and comparatively large movements of their body after implantation of granulated bone grafts compared to large animals [104,105]. Subcutaneous tissue in large animal provides relatively lesser movement of the subcutaneous tissue environment for implanted bone granules, which may be favourable for ectopic new bone formation. Another reason might be the increased stability of block scaffolds themselves compared to granules. Maybe granules tend to move easily with the environment, whereas block grafts remain stable and allow only micromovement on their surface. This might explain the increased bone formation on the surface around block scaffolds [74,87]. The micromovements might be too macro for osteocytes or stem cells to produce bone extracellular matrix in granules.

Recent development in tissue regeneration provided biomaterials for slow release of proteins over a period of time, thus giving cells continuous stimuli to differentiate into desired cells [60,65,66,68,70,73,75,76,78,92,98-100,109,116-121]. The level of gene expression in the surrounding tissue to regenerate bone can be enhanced by delivery of plasmid DNA using hydrogel microspheres, more than plasmid DNA solution. BMP₂-incorporated gelatin/ β -TCP scaffolds lead to significantly increased osteogenesis compared to the chem-

ical induced osteogenesis in specimens [60,65,66,68,70,75,76,78,92,98-100,109,116-121]. In previous studies, various groups showed a continuous release of BMP in vitro and in vivo [65,66,68,70,73,75,76,92,98-100,109,119-121]. This suggests that the environmental chemical or humeral factors play an important role in the formation of bone and that bone formation can be orchestrated when using these factors.

In our table different animal model have been shown to support various sorts of bone regeneration. This different bone regeneration suggests that the different animals provide different chemical and humeral environment for cells, thereby influencing bone formation enormous. In addition, the site of implantation might also affect bone formation by providing different humeral or chemical environmental factors [12,24,79-92,105].

So far, regular X-ray examination, CT or quantitative CT scanners have been used to assess trabecular bone structure and bone mineral density. Good correlation between measured density values of trabecular bone and biomechanical properties have been demonstrated [79-81]. Although CT resolution has improved over time, three-dimensional evaluation of new bone formation requires different high-resolution methods. Recently ultra-high resolution volumetric CT scanning (VCT) has been shown to correctly analyse bone formation in tissue engineered constructs over the course of six weeks [22-32,60,65,66,68,70,71,73,75,76,92,98-100,109,116-121]. A different approach to estimate the formation of new bone in small animals is the use of micro-CT scanners. However, a clinical application of these micro-CT scanners is very limited [22-27,65,66,68,70,71,73,75,109,116,117,119,120]. But bone formation has to be concordant with radiographic imaging, as it was shown previously [71].

Histologically bone formation can be monitored. Here, regular histology must be differentiated from histochemistry or immunohistochemistry. All are being used to show bone formation, integration and osteoactivity. However, the use of the method depends on the aim of the researcher. Bone should also provide mechanical stiffness and withstand torsion. For evaluation of new bone formation, osteointegration into defects and mechanical strength ultimate tensile strength (δ UTS), failure strain (δf), fracture energy (E_f), and the dynamic tensile modulus (M) can be tested biomechanically [2,5-13,18,22-32,60,65,66,68,70,71,73,75,109,116-120].

Using microscopy, structural changes can be monitored. Here, various methods have been applied such as scanning electron microscopy, transmission electron microscopy or energy dispersive X-ray spectroscopy. Using light microscopy slides have to be stained to evaluate presence of bone. Regular histopathological staining for bone are van Kossa, alcian blue or alkaline Phosphatase, whereas the last is usually stained within the cells themselves and not within the extracellular matrix [22-32,60,67-69,71,73,75,90-102,105,109,116-121]. When applying the scanning microscopy, tissue is characterised morphologically, transmission electron microscopy allows for ultrastructural morphological evaluation [22,60,75,90,105,116-120]. However, the tissue has to be prepared special, but structural analysis shows trabecular formation, integration into the surrounding bone stock or allows for very early proof of non-union. In combination with physiochemical characterization by dispersive X-ray spectroscopy analysis can be complete [82-85]. The drawback of microscopy is the invasive method to harvest bone or tissue material. Ideally, all methods are combined [71].

In conclusion, it is important for any animal study that many factors

should be taken into account, when selecting the suitable species, the desire defect model and methods for evaluation.

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