RODENT BONE EXPERIMENTAL MODELS AS A TRANSLATIONAL TOOL FOR BIOCOMPATIBILITY TESTING OF NEW BIOMATERIALS

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Abstract. In vivo experimental models are a key factor for the study of normal and pathologic tissue regeneration. This requires the use of advanced site-specification bone defect in small animals. Nowadays rodent models are comparable directly to the standards in large animal models and humans due to advances in ostheosynthesis techniques. This review is discussing the most available and commonly used bone defects (mandibular defects, calvarian defect and large bone defects) in laboratory animals (mice, rats) for testing various biomaterials (polymers, ceramics, cells, etc.). These site defects are the most frequently used to test innovative biological biomaterials as bone substitutes. It further describes procedures, methods, clinical exams, paraclinical exams (imagistics: CT, micro-CT, morph metric analyze, biochemistry) and histopathological results from various studies that can help attest the biomaterials performance and respect the wellbeing of animals. These models are used for the testing of biocompatibility, toxicity and osteointegration of a biomaterial at the locus of bone deficiency. Thus, in vivo bone defects are essential tools for certifying the biocompatibility, biophysical effects and biosafety in using biomaterials in regenerative medicine.

Key words: rodent models, bone defect, biomaterial, ostheosynthesis, biocompatibility

INTRODUCTION

In vivo bone defects certify the biocompatibility, mechanical resistance and safety in using biomaterials in regenerative medicine (Li et al, 2015). The defects allow new synthetic materials to be tested and used as implants. The majority of the experimental defects are realized on rats or mice (45%) and rabbits (35%). 20% are realized on sheep, goats and pigs (Gomez et al, 2011). In some cases, for the use of the biomaterial in human medicine it is necessary in vivo testing both in small animals and big animals (Peric et al, 2015).

Based on the dimensions, surgically realized bone defects are classified in 2 categories: critical and non-critical defects, critical defect meaning the defect that does not heal during the natural life span of the animal, not being possible the union of the bone. Non-critical defects can heal naturally, the bone regaining its integrity in a period of time (Garcia et al, 2013). ISO 10933-6 recomands using non-critical defects in small animals based on the type and model of the implant (Spicer et al, 2012). For example, a screw type implant can have between 2 and 4.5 mm in diameter and no more than 6 mm length and a cylindrical implant cannot pass 2 mm in diameter and 6 mm in length. Also the number of defects is limited; there should not be more than 6 defects per animal (Spicer et al, 2012).

The purpose of this review is to highlight the most available and commonly used bone defects (mandible defects, calvarian defects and large bone defects) in laboratory rodents (mice, rats) for testing various biomaterials (polymers, ceramics, cells, etc.). These

site defects are the most frequently used to test innovative biological biomaterials as bone substitutes (fig. 1). It further describes procedures, methods, clinical exams, preclinical exams (imagistic: CT, micro-CT, morph metric analyze, biochemistry) and histopathological results from various studies, that can help to test the biomaterials performance and respect the wellbeing of animals.

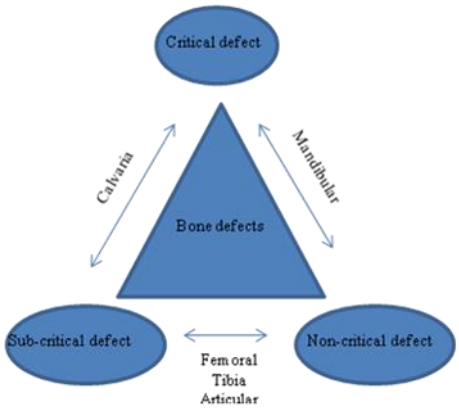

Fig. 1. Bone defect models characterized by gravity and the locus of deficiency

MANDIBULAR DEFECT

Mandibular defects are realized for testing materials used in oral and maxillofacial surgery (Briebly et al, 2016). These defects offer the surgeons the possibility to study new materials for implants, dentures, bone regeneration after tumor resections (squamos carcinoma, chondrosarcoma, osteosarcoma, fibrous dysplasia, juvenile osifiant fibroma) or other facial pathologies (DeConde et al, 2014). The tests are meant to regain the form, the bone function and at the same time the mastication (Peric et al, 2015). The majority of the studies are realized on gnawers and rabbit's mandible, using 1, 2, 3 and 5 mm defects in the monocortical mandibular bone, or 1 and 2 mm defects in the proximal body of the mandible. The 5 mm defect is a critical defect in rodents, needing to place fixators for full regeneration (DeConde et al, 2014, Brierly et al, 2016). The mandibular defects are realized by using a micromotor with low speeds (3000-4000 rpm), after the superficial layers were previously incised: skin, the masseter muscle and the periost. Non critical bone defects are treated with different polymeric biomaterials or ceramics filled with hydroxyapatite (Xue et al, 2009), nanoparticles (Rajendran et al, 2015) and grafts combined with stem cells (Wang et al, 2016), adipocytes or osteogenic proteins (Zhang et al, 2016). Critical bone defects are realized for the study of osteocutaneous grafts, plates, screws, gels or foam with growth factors or cells which strengthen the defect locus and providing osteogenic cells (Ren et al, 2005, Ren et al, 2007). The data are correlated at different time intervals, based on the material and the scientist option, starting with the results quantified between 2 and 32 weeks. Preclinical analyzes most frequently used are CT and micro-CT, but the gold standard is the histopathology exam (An et al, 2003).

BIOMATERIAL UTILIZATION IN MANDIBULAR BONE REGENERATION

A polyhydroxybutirate membrane was tested on a mandible bone defect in rats. The membrane was inserted in a circular defect with 2 mm diameter and 3 mm depth on one side

of the mandible. The polymer membrane was sutured at bone level with resorbable thread through small holes previously made in the bone. At first an intensive inflammatory exudates, fibrous tissue and blood clots in both bone defects were observed. The results showed that starting with day 90, the bone defects treated with the polymeric membrane healed, bone tissue filling the defect up to 62%. The healing in the control groups took place with the defect being filled with connective tissue (Kostopoulos et al, 1994). Ren et al, 2005 studied in vivo regeneration of a mandible defect in rabbits treated with porous graft made from PLGA. A critical bone defect with 12 x 15 mm size was induced and 3 experimental groups treated with PLGA, PLGA and stem cells, sham surgery group were formed. The animals did not show macroscopic inflammation signs, abscesses or infections which demonstrated on one hand the biocompatibility of the graft. The animals were euthanized at 6 and 12 weeks. The study results showed a complete degradation of the polymeric graft after 12 weeks in both groups treated with polymers. Additionally, in the group treated with PLGA alone were signs of bone regeneration with new bone tissue at the edge of the defect, the union not being complete in this case. Full regeneration with the union of the defect edges by bone tissue was achieved in the group treated with PLGA graft and stem cells. (Ren et al 2005). The same experimental protocol was used for testing 2 polymeric grafts based on PLA-PEG and PLGA and the same grafts coated with stem cells. The study was comparative between the biocompatibility and bone regeneration ability of the materials. Beside the histopathology exam it was also used SEM analysis of the rabbit mandibles after the death of the animals. An osteogenenic index based on the osteogenesis area and the section area (OY= (OA/SA) x 100%) was calculated. After 12 weeks, the results showed that all groups treated with polymeric grafts and stem cells achieved a full regeneration with total absorption of the polymeric material. The PLGA and stem cells graft showed the highest biocompatibility and the osteogenesis index was far superior in the group mentioned above than in the PLA-PEG and stem cells group (72.7%) and PLA-PEG groups (Ren et al 2017). Another study for testing a graft composed of PLA with hydroxyapatite (HA) and collagen nanoparticles coated with stem cells from human alveolar bone was realized. A 10x5x3 mm critical defect in 30 rabbits was performed. The animals were divided in 5 groups treated with PLA and HA nanoparticles not coated, nanoparticles coated with alveolar cells, PLA and nanoparticles coated with alveolar cells cultured in osteogenic environment for 7 days, a positive control group with the same graft and bone cells from the iliac bone and a negative control group not treated. The animals were euthanized after 8 weeks. Morphometric analyzes of the histopathological samples and microCT analyzes with the determination of volume and bone density and trabecular microarchitecture were made. The histopathology results revealed regeneration present in groups treated with polymeric graft and nanoparticles coated with alveolar cells. It was observed the presence of mature bone tissue and an abundance of osteoblasts at the surface, the group being considered the one with the highest regeneration rate (Wang et al, 2016).

CALVARIAN DEFECT

The calvarian bone defect model is suitable because of the possibility of creating a uniform defect, easy evaluated through simple radiographic methods and histopathology (Gomez et al, 2011). In the same time, the anatomic position makes the surgery facile. Implanting the studied materials doesn't need the use of external or internal fixators neither in critical or non-critical defects (Gomez et al, 2011). The only disadvantage discovered during the studies is the impossibility of testing heavy biomaterials which causes a major

mechanical stress (Shirasu et al, 2009; Suenaga et al, 2015). The majority of the studies on calvaria defect were successfully realized on rodents. Non-critical defects are considered to be the ones smaller than 3 mm, the sub-critical defects are between 3 and 5 mm and the standardized critical defect has 8 mm in rats (Hollinger et al, 1990, Spicer et al, 2012). In mice 0.8, 1 and 1.5 mm defects are used, the last one being the critical one. Usually there are produced 2 or more calvarias defects, one of them being considered the control defect (Spicer et al 2012). The experimental protocol consists of incision of the skin, the decollation of the periosteum and the creation of a defect with the help of a dental micromotor used at low speed. The bone is not completely penetrated avoiding the lesion of the Dura mater and underlying blood vessels. Complementary exams as RX, CT, micro CT and SEM are used (Kallai, 2011). The histopathological analyze remains the universal standard accepted in this case also (An et al, 2003).

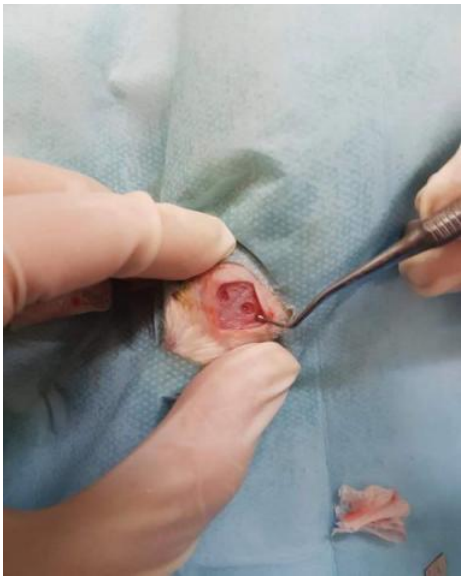

Fig. 2. The calvaria bone surgery, a created defect of 2 mm wide in Wistar rat

BIOMATERIAL UTILIZATION IN CALVARIAN DEFECT

Shirasu et al, 2010 used a powder made from calcium triphosphate granules combined with bone marrow cells from human tibia. The animals underwent surgery, being created a 4 mm calvarian defect. 4 groups were made, one with bone marrow and calcium triphosphate graft, one with only bone marrow, one with only calcium triphophate and one left untreated. Histopathology and imunohistochemistry were made (PCNA expression, Runx2, OPN, TRAP). At 5 and 10 days post surgery no new bone tissue was found. After 20 and 30 days the complete filling of the defect with osteoblasts and trabecular bone was seen only in the group treated with bone marrow and calcium triphosphate graft. In the group treated only with bone marrow, a beginning of bone regeneration was observed, but the defect was not filled. In the other groups, new bone tissue was not formed (Shirasu et al, 2010). Suenaga et al, 2015 tested the regeneration capacity of stem cells spheres comparative with stem cells combined with calcium triphosphate granules and calcium triphosphate granules alone. 8 mm defects were realized on nude rats. The left defect was treated with the materials mentioned above and the right one was considered a control defect. In this way 3

groups were tested. The animals were humanly euthanized after 8 weeks. Histopathology and imunohistochemistry exams by detecting osteocalcin and osteopontin, microCT exam for the 3D reconstruction of the defects and Raman spectrophotometry for detecting the spectral characteristics of the new bone tissue comparative with mature bone were performed. The results of the histopathology and imunohistopathology analyzes showed the osteogenerating capacity of simple stem cells, without the calcium triphosphate. The persistency of the phosphate granules in the defect after the 8 weeks was also observed. The microCT revealed the formation of new bone tissue using the stem cells, but without being present in the centre of the defect, and the Raman analyze showed a mechanical resistance and low elasticity of the newly formed bone (Suenaga et al, 2015).

LONG BONE DEFECT

There are numerous experimental models of fractures or bone defects on femur, tibia and ulna in mice, rats and rabbits. The models targeting long bone diaphysis are classified based on the union capacity of the bone edges (Cheng et al, 2013). Therefore, there exists the union model, delayed union provoked by pathologies as osteoporosis or experimental infection with bacteria, diabetes or obesity (Garcia et al, 2008, Garcia et al, 2009). Delayed union can be also obtained through the lesion of periosteum, of blood vessels or of the nearby soft tissue. The non-union model consists in the impossibility of uniting the bone edges in a period of 15 weeks in rats and 12 weeks in mice. The critical defect is the model in which there cannot be a union without treating the defect with grafts or biomaterials (Kirker-Head et al, 2007). At the moment, the size of the critical defect in nude mice is 3 mm (Zwingenberger et al, 2013). At the same time, it was stated that the critical defect for these models in long bones is 1.5-3 times higher than the bone diameter (Garcia et al, 2013). The healing process is influenced also by species, age and gender. The majority of the critical defects created on the diaphysis need external or internal fixators. There are also used femoral epiphysis defects because are easy to perform, offer a greater stability for the material and do not require fixators (Garcia et al, 2013). These complex models are suitable for testing numerous composite materials from gels, powder, cells to grafts realized through 3D printing.

BIOMATERIAL UTILIZATION IN LONG BONE DEFECTS

The first studies, which confirm the strong osteoinductive effect of calcium citrate powder, are realized by Zhang et al, 2012 on femoral bone non-critical defects of 1.2 mm, 1.5 mm, 2 mm and 2.5 mm in rabbits. The 4 defects of different sizes were created on both legs, in one leg the defects were filled with calcium citrate and in the other leg the defects were considered control. The defects were analyzed during the experiment with the help of radiographic images. It was demonstrated that calcium citrate had a positive effect on the first period of bone regeneration in defects smaller than 2mm (Zhang et al, 2012). Mohan BG et al, 2014 studied the effects of a graft made from strontium and calcium phosphate on a 1.5 cm critical ulnar defect in rabbits. The animals were sacrificed after 4 and 12 weeks. For determination of the osteointegration and the regenerative capacity of the grafts were realized histopathology analyzes, microCT, morph metric and radiographic analyzes. The formation of bone tissue was directly proportional with the material degradation at 12 weeks. Due to the radio-opacity offered by de material it was decided the importance of using it in the reconstructive surgery (Mohan et al, 2014). Fugita et al, 2011 tested the controlled release of some osteoformating proteins embedded in a jelly sponge in comparison with a simple

sponge or jelly sponge with tricalcium phosphate in a 4 cm ulnar defect in rabbits. The study showed that the regeneration did not occur in groups with the materials without the osteogenic proteins (Fugita et al, 2011). Other studies had as subject the natural capacity of bone regeneration in osteoporotic rats comparative with normal rats. The authors created a 3 mm femoral defect which was evaluated during the study with the help of contrast PET-CT. Osteoporosis was induced through ovariectomy. The study demonstrated that the healing rate in osteoporotic animals is low and this could be a good study model for delayed regeneration. (Cheng et al, 2012). Raquez et al, 2011 studied a composite material made from PLLA and pseudowollastonit, a ceramic material. The study targeted introducing cylindrical composites in 4 tibia defects with 1 mm diameter and 2 mm depth in rats. The evaluation was made through micro CT and histopathology. The scientists observed that the defect which was not filled with composite material healed during the 5 weeks of the study. On the other hand, the defects treated with the composite materials din not heal totally because of the low degradation rate of the material. The histopathology analyzes reveled bone tissue at the edge of the defect, the conclusion being that the composite material is osteoinductive (Raquez et al, 2011). Another study revealed the success of treating femoral osteochondric defects in rats with grafts made from PLGA nanoparticles and hydroxyapatite combined with stem cells. The created defects were 1.5 mm in diameter and 3 mm depth. After 12 weeks the animals were humanly euthanized. In necropsy the complete healing of the defects was observed. The histopathology exam showed chondrocytes and imunohistochemistry exam showed the presence of collagen type II and the absence of collagen type I, which suggested the generation of cartilaginous hialin tissue with the absence of fibrocartilaginous tissue (Xue et al, 2010).

CONCLUSIONS

The experimental models mentioned in the current study are used for the testing of biocompatibility, toxicity and osteointegration of a biomaterial at the locus of bone deficiency. In vivo bone defects are essential tools for certifying the biocompatibility, biophysical effects and biosafety in using biomaterials in regenerative medicine.

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